

Spying is getting out of hand

State Department allegations of Soviet surveillance in Moscow will cause much more trouble than the technique can have been worth. The time has come to agree a code of conduct for spies.

JUST as science fiction has a tendency to become fact, the literary genre of the spy thriller is turning into a curious kind of reality. So much is clear from the US State Department's claim last week that the members of its embassy's staff in Moscow have been contaminated with a chemical (nitrophenylpentadiene) which can be made to be fluorescent by irradiation in the ultraviolet. The allegation, on which further details are promised, is that the material has been planted in people's clothing and on, say, the steering wheels of motor cars in such a way that drivers' hands are likely to be contaminated. The announcement lends credence to the idea that, by means like this, it would be possible for Soviet security authorities to learn what members of the US embassy are about during the course of their working day.

There are several things to say, of which the first is that such a practice would be a serious infringement of the rules that should govern the conduct of diplomatic missions, laid down by the Vienna conventions on the subject. The principle is (or should be) that even people who work as diplomats in foreign countries are, like their hosts, entitled to a measure of personal privacy and to the presumption that they are not criminals — unless evidence to the contrary comes to light. That is why the agreements governing the conduct of embassies usually allow for people's freedom to travel within a certain well-defined area, and entail the assumption that meetings with local residents are by definition innocent unless discovered to be otherwise. A little reflection will show that the whole business of international diplomacy would grind quickly to a halt if these assumptions were not valid. Seeking to provide continuing surveillance of what foreign diplomats are up to, as the Soviet Union may have been doing, would be a serious breach of these important principles.

But even if the allegations are borne out by further evidence, what in principle will have been accomplished? The use of chemicals fluorescent in the ultraviolet has a minor place in forensic science as a means of marking objects whose origin is known, but a more conspicuous place in the luxuriant detective-story literature of the West. The obvious difficulty, in the scheme the Soviet authorities are said to have used to track the movements of members of the US embassy in Moscow, is that there is no obvious way in which generalized use of the fluorescent chemical could possibly have been of value to them. It is unthinkable that the authorities could have scoured Moscow with ultraviolet lamps, looking for traces of US diplomats whose clothing had been doped with the tracking chemical. What would be possible is to tell whether some otherwise unknown person seen seated in some public place had been a US diplomat, or whether a particular high-denomination currency bill (a dollar or a rouble) had been peeled off a stack of notes by a person carrying the tell-tale chemical on his fingers. In other words, if such a chemical has been used and has been found

useful by Soviet counter-intelligence authorities, it is more likely that its function has been to help tell what Soviet citizens have been doing than to keep a permanent watch on the movements of US diplomats. But the chances are that a technique like this would have been of only marginal value in the legitimate prosecution of counter-intelligence by the Soviet authorities. If real, the allegations probably point to some over-zealous and impracticable scheme suggested by an over-enthusiastic committee.

Even if diplomats elsewhere are unlikely to be exposed to hazards of this kind, because other police authorities are unlikely to copy the bizarre technique now described, there is not much else that is cheerful at this stage. If the details of the allegation are borne out, they are bound to sour the climate in which the Soviet Union and the United States approach the next few critical months of conversation about arms control, and about their general relationship with each other. That is the opposite of good news. In the United States, thrown into anxiety about national security by recent cases of espionage and by fears that foreign nationals, diplomats and others increasingly function as espionage agents, the result of the disclosures from the Moscow embassy may be to amplify still further the demand for extra restrictions on the movement of people away from the places to which they are formally posted. And the result of that would be a further twist in the depressing game of tit-for-tat that has characterized the relationship between the Soviet Union and the United States for too long now.

So, in a sense, the best hope is that the allegations now made by the State Department turn out to be untrue, or that while the details may be verified, the implication that chemicals have been used as a means of keeping track of diplomats may be mistaken. Unhappily, that is unlikely to be the case. But there is a chance that the Soviet leadership will be able to argue, with reasons given, that any such practices were carried out without its knowledge, perhaps by junior people in some over-eager part of its establishment. Even that would be a great deal to ask for; the Soviet Union has never been happy to confess to imperfections in its way of running its own affairs. Yet this, it seems probable, will be the only escape the government of France will have from the allegations in New Zealand that French agents were somehow involved with the sinking of the Greenpeace vessel at the end of June.

Espionage and its elaborations (sinking ships, assassinating people) may seem to governments necessary to their domestic security but they are also a threat to international security. Is it entirely unthinkable that they should be regulated by international treaty? The obvious difficulty is that all governments engage in espionage but insist that their hands are clean. That is increasingly a sick joke. Moreover, there are at least some circumstances in which the reality of espionage is acknowledged, as when governments trade groups of captured agents at the Berlin Wall or elsewhere. Why not go the obvious step further, and negotiate a kind of code of conduct for intelligence services. An agreement not to take direct action against third parties (such as Greenpeace) would not, after all, constitute an admission that other less foolish practices are matters of routine. President Reagan and Mr Gorbachev might usefully spend a few minutes on the topic at their meeting in November. □

Biological manuscripts

Contributors are reminded that, with the transfer of the Biological Sciences Editor to the Washington office, they should in future send **four copies of all manuscripts** offered for publication either (as at present) to London or to Washington.



Upsetting apple-carts

The British research establishment is ripe for a shake-up. Here is one recipe.

THE past year has been a bigger trauma for the British research community than even its immediate predecessors, dominated as they were by the dwindling value of the funds available for research. Now, perhaps not before time, structural change is in the air. Several unrelated issues have come to the boil at the same time. The Science and Engineering Research Council, with a new chairman and a substantially changed membership (see p. 756) must soon produce a plan for the years ahead that will give the largest of the research councils a coherence that will hold it together in the face of centripetal tendencies born of its complexity. The University Grants Committee is committed to a plan for concentrating financial support on the universities strongest in research, but neither the committee nor its dependants know how the system will work. The Advisory Board for the Research Councils (ABRC) is busily putting out fires where it can find them, and is for example embarked on an inquiry into the plight of the British Geological Survey, the largest single component of the Natural Environment Research Council (NERC), but seems not yet to have attempted the more urgently needed inquiry into its own constitution.

The immediate effect is that people in laboratories have been painfully reminded of the fate that has already befallen some thousands of researchers and academics, whose jobs have melted away in the past few years. But as the whole world knows, there is nothing so demoralizing as uncertainty. It is in everybody's interest that the changes now under way should be carried through quickly. Things have reached the point at which almost any decisions would be better than the continuing indecision that seems to grip the establishment. There is nothing new in the recipe for change that follows, most of which has been advocated in the past year or more. Other recipes might serve the same purpose. The need is that somebody should pick one of them and stick to it.

Money. The government has kept its promise of five years ago that research council budgets would stay constant, and there has even been some extra money for earmarked purposes such as information technology. It is intolerable that there should have been so little substantial help with the reorganization of laboratories forced on the Agricultural and Natural Environmental Research Councils in the past few years, for the government has thus allowed what it considers to have been past mistakes to cast a shadow over the health of the whole enterprise. But people had better learn to live with the notion that there will be no extra funds. But reorganization is necessary for other reasons than financial shortage.

Management. The touchstone for the past dozen years in the management of British civil science has been the Rothschild principle, by which government departments, proxy "customers" representing the interests of the end users of research, commission much of the work carried out in civil research establishments. That principle is now dead, as shown by the ease with which government departments have been able to walk away from research institutes they had previously supported. (The British Geological Survey is that most conspicuously left high and dry by departmental fickleness of this kind.) So there is a need for other mechanisms for supporting important and creative work. To detach some parts of the publicly supported research network altogether from the government would make sense. ("To privatize" is the fashionable verb.) The Plant Breeding Institute, perhaps the most immediately productive of the agricultural research council's establishments, is the obvious candidate if it were allowed also to enjoy the income generated by its findings through the National Seed Development Organization; why does nothing happen? But if the government hopes that the cost of public research could be drastically reduced by hiving off large chunks of it, that ambition should be suppressed. Nor is there as much hope as there may seem in

schemes for encouraging parts of the research enterprise to compete for research contracts with private industry; the universities and private research organizations are now eagerly exploiting that market, the former at cut-price rates. Deciding what parts of the public research network to keep and to cherish, and what parts to close down, is an urgent need. Since many of the neglectful government departments are probably mistaken in their indifference to research, the British government corporately should take a view on the wisdom of transferring organizations such as the institutes responsible for geological survey and oceanography back to where they came from (the Department of the Environment and the Royal Navy, respectively).

Leadership. One of the contributory causes of the present decline of British science is that working scientists no longer have a sense that there is a powerful and influential group of people looking out for the well-being of their work. The heads of the research councils are mostly reasonable men, but are undemonstrative and, by tradition, are also anxious not to give the impression publicly that they seriously dissent from the way in which science is being managed, or mismanaged. The result is that they seem to their constituents more like civil servants than the spokesmen of their subcommittees. In the peculiarly British environment, the Royal Society might most suitably play this role. But would the task of helping to point the scientific enterprise in the right direction be feasible within the convention of politeness to which the society is committed?

Institution. Many of the organizations responsible for the direction of civil research in Britain have outlived their usefulness, or have reached the point where only substantial change will fit them for what emerges as their purpose. ABRC itself is a point at which reform should start. The council is too large to be a workable committee, and too representative of conflicting interests to be an effective way of settling contentious issues. To the extent that the council's function is to advise the Secretary of State for Education and Science, one man would suffice. To the extent that it is necessary to put forward the opinions of the users of what the research councils provide — that there should be some predetermined pattern of spending — a handful of outsiders would be the ideal. To the extent that the council should have a part to play in helping to understand what is happening to British research, there is a need for a more effective secretariat than at present. All this is roughly what Sir Douglas Hague, the chairman of the Economic and Social Research Council and one of the members of ABRC, was saying when he joined the council two years ago. Why have his fellow members not listened to what he was saying? And why have the same people not understood the case for disbanding NERC, the research council that had the bad luck to be set up with terms of reference too ambitious for the components with which it was endowed.

Coordination. One of the chief diseconomies in the present organization of research in Britain is that there is no way in which the different parts of the research enterprise can be meshed with each other. Defence research is an entity on its own (but change may be on the way). And separate government departments continue to maintain laboratories and research institutes which become, as morale sinks deeper, more separate from each other than they have ever been. The National Physical Laboratory, dependent on the Department of Trade and Industry, has been in its time a powerful stimulant of research in universities and elsewhere. It is still a worthy place, but much less a laboratory to be proud of. That, too, is a field ripe for reform.

On the face of things, it would be for the government to put these things to right, just as it has fallen to the government to decide what should next be done about the renegotiation of the British relationship with the European high-energy physics laboratory (CERN) at Geneva. But no doubt from depression, the country's capacity to make decisions seems to have been undermined. The notion that any decision is better than indecision is, of course, dangerous; it provides a licence for foolishness. That is why the scientific community itself should find some way of taking charge of events, such as they are. □

Ballistic-missile defence

US under pressure from Europe to share data

Washington

Now that negotiations on allied participation in the Strategic Defense Initiative (SDI) have begun in earnest, the US government and the Pentagon are coming under strong pressure from the British and West German governments to grant full access to technical know-how gathered in the course of the development as part of the price of overseas participation. But the British government has also raised the possibility that participation might not be worthwhile unless the value of the contracts awarded to British companies and research organizations reaches some predetermined value — \$1,500 million has been mentioned, to the consternation of some administration officials.

The report that the British government is asking for contracts worth at least \$1,500 million appeared last week in the *Baltimore News* newspaper and has been obliquely confirmed. But British officials have said that any figure mentioned at this early stage should be regarded as an "opening bid", rather more an indication of the scale on which the Pentagon should be thinking than as an ultimatum.

Negotiations appear to be at a relatively early stage, with the arrangements for dealing with the security of classified information during the research phase of SDI agreed but not yet formally adopted. One of the points made by British negotiators is that any involvement of British companies as contractors for SDI will involve the British government in supervision and monitoring on a substantial scale, the cost of which would be far from negligible. But the chief concern is that companies or other research entities involved with SDI should not be dealt with as mere research contractors and recompensed on the basis of the time and trouble expended, but that they should be genuine partners in the parts of SDI in which they are engaged.

SDI is likely to centre on fields such as infrared sensors (the basis of boost-phase detection) and signal-processing, in both of which British defence research establishments and associated defence manufacturers have some expertise. One of the British demands is that there should be no restraint and British companies should not be at a disadvantage, compared with US participants, in the later exploitation of developments in which they have been engaged.

A more distant and more difficult issue, raised chiefly by the government of West Germany, is whether governments and national companies participating in SDI should also have access to the later com-

mercial exploitation of technical innovations in which they have not been directly involved.

Even though these questions have not yet been decided, the United States

appears to be in some haste to bring the negotiations to a conclusion. With the sequence of diplomatic meetings planned for next month and thereafter, the bilateral summit in November chief among them, there are obvious benefits in being able to announce allied participation in SDI. No doubt the potential partners have made similar calculations. Just what price will be struck for the political advantage and the technical assistance may be the kind of question the game of poker is designed to answer. □

UK overseas students

Market forces shrink demand

FIVE years after the British government's decision to require overseas students to pay a full "economic" rate for their college or university education, the numbers are still in decline. A report from the Commonwealth Secretariat estimates that the number of overseas students has dropped to some 55,600, a fall of more than 30,000 since the introduction of full-cost fees in 1980, including a fall of 1,000 last year.

Despite a declaration from Commonwealth education ministers meeting in Nicosia (Cyprus) last year that it is important to foster the exchange of students between member nations, the Commonwealth Secretariat notes that exchanges within the Commonwealth are on the decline, but that elsewhere student mobility is increasing. West Germany now accepts 72,000 foreign students and appears to be taking more students from Commonwealth countries no longer able to send as many to the United Kingdom — including Cyprus, Tanzania and Kenya.

The United States remains by far the largest host nation, with some 340,000 foreign students. France has more than twice as many as the United Kingdom (128,000) and the Soviet Union has 82,500. Japan, at present with some 10,000 foreign students, plans to expand this to 100,000 by the end of the century.

The foreign policy implications of providing places for overseas students were recognized by the British government in 1983 when, after considerable pressure from the Commonwealth (most vociferously from Malaysia), the then Foreign Secretary Mr Francis Pym announced a three-year £46 million package to subsidize overseas students. The "Pym package", now entering its last year, singles out certain countries as specially deserving cases—Cyprus, for instance, with a large refugee population, no major university

and strategic importance to Britain — as well as potentially influential individuals from potential trading partners.

The clear determination that the United Kingdom should get "value for money" for overseas students supported financially underlines the economic problem of fostering an exchange between the developed and developing nations. With a brief that the mobility of students between Commonwealth countries is a good thing no matter in which direction the movements take place, the committee responsible for the report suggests that the less developed countries with relatively sophisticated university systems might be encouraged to take increasing numbers of students from countries with few universities.

Not only the supply of university places is affected by economic pressures. Shortage of foreign currency means that the demand is also depressed in countries such as Nigeria and Malaysia, even if the cost of education in host nations is not increasing.

In the immediate future, the current trend in Britain seems likely to continue, as fees are due to increase by a further 5 per cent for the year 1985–86. But for the long term, the committee raises the possibility that student exchanges may not be indispensable for sharing the access to high-level institutions. New information technologies, says the Commonwealth Secretariat, should be considered as an alternative. If the best learning in the world is available at the nearest computer terminal and telephone line, will there be any need for the students to stir from home?

Charles Wenz

"Commonwealth Student Mobility: A Way Forward", is the fourth report of the Commonwealth Standing Committee on Student Mobility (Commonwealth Secretariat, Marlborough House, London SW1 5HX, UK).

Foreign students in higher education in Britain, 1979–80 to 1983–84

	1979–80	1980–81	1981–82	1982–83	1983–84
Commonwealth	43,630	38,718	32,710	28,016	27,370
Non-Commonwealth	44,407	38,912	31,361	28,649	28,238
Total	88,037	77,630	64,071	56,665	55,608
(Commonwealth as % of total)	(49.5%)	(50%)	(51%)	(49%)	(49%)

Source: British Council

Australian budget

Modest gains for science

Canberra

THE federal government's budget, made public last week, allows modest gains for the government's chief science agencies at a time when the general theme is that of restraint. Indeed, with inflation between 7 and 8 per cent a year, the Hawke government plans to cut its overall deficit to just under \$A5,000 million, some 2 per cent of the estimated Gross Domestic Product for 1986.

According to science minister Mr Barry Jones, the budget for the science portfolio will increase next year to a total of \$537 million, an increase of 6.4 per cent. Within that total, however, there are small but significant shifts of emphasis. The largest single item, the budget of the Commonwealth Scientific and Industrial Research Organization (CSIRO) will be \$A336.7 million which, after allowing for inflation, represents a small cut. No doubt the issue will be reviewed after the appearance of the report on the organization by the Australian Science and Technology Council (ASTEC) expected later in the year.

The chief (if modest) winners from the new budget are the Australian Research Grants Scheme (ARGS), whose budget increases to \$A27.5 million, a rise of 10 per cent. Both the Weather Bureau and the Antarctic Division will have increases of the same size.

Perhaps more significant than these small changes, Mr Paul Keating's budget includes provision for increasing the numbers of students in tertiary education by 20,500. As yet, no decision has been made about the distribution of these places among the three sectors of higher education, but the government announced last week that it had accepted the general arguments of the plan for the reorganization of the Commonwealth Tertiary Education Commission, put forward a few months ago by the commission's chairman, Mr Hugh Hudson. Among other things, Mr Hudson advocated closer integration and liaison among the three sectors.

One unexpected feature of the new budget is that the funds available to the Australian Industrial Development Research Board next year will remain \$A65 million, a reduction of only \$A2 million on this year's budget. It had been expected that these funds would be sharply reduced to offset the plan to introduce, from 1 July next, a 150 per cent tax credit so as to provide manufacturing industry with an incentive to undertake more research and development with their own funds.

For the Australian research community, as for others affected by the budget, the biggest uncertainty for the months ahead is the way in which the economy will respond to the changes that have been made. Mr Paul Keating's plan to engineer

a shift from direct to indirect taxation has now been abandoned. The government was boasting last week of the comparatively rapid economic growth of about 4.5 per cent a year, promising more of the

British research

Bed of nails at last vacated

SIR John Kingman, who has occupied the most conspicuous bed of nails in the British research establishment for the past five years, as chairman of the Science and Engineering Research Council (SERC), leaves this week knowing that his council still has to hammer out a policy for the future. Although impending shortages of



funds sent the council and its staff into an urgent review of priorities last autumn, the resulting "corporate plan" has seemed to the council at its two most recent meetings to be more a list of options than a set of decisions. It will now be for the new chairman, Professor E.J.W. (Bill) Mitchell and the new council (which will have no fewer than eight new members when Mitchell's own place is filled) to decide what lies ahead (see *Nature* 15 August, p.568).

Kingman will become vice-chancellor of the University of Bristol at the beginning of next month. Of the British research enterprise in the past five years, he acknowledges that the British government has kept its promise to keep research spending constant, but that the parallel decline of university budgets had been a serious setback. Now, he thinks, the signs that the rate of emigration of able scientists from the United Kingdom has accelerated again shows that the chickens are roosting uncomfortably.

Politically, there are some hopeful signs. Kingman says, for example, that several Members of Parliament are now more sympathetic than they used to be and conscious that Britain differs from competitors such as the United States and France in attempting to keep science spending constant.

But he also considers that the British

same to come.

But Mr Keating has also been giving solemn warnings that the inflation rate could be sharply increased if wage settlements seek too much compensation for past inflation. Since the budget spending items now announced are fixed in cash terms, their value could be seriously affected by the inflation rate. □

scientific community has been churlish and tactically unwise in not acknowledging more fulsomely that its paymaster, Sir Keith Joseph, Secretary of State for Education and Science, had made a brave political effort to secure extra research money at the expense of student grants at the end of 1984.

Largely for financial reasons, Kingman's spell at SERC has been the most uncomfortable since the reorganization of the 1960s. It has also been a period of indecisiveness. Even major questions such as the council's future commitment to high-energy physics remain undecided and are likely to be out of the council's hands at least until the British government has decided how a renegotiation of the CERN agreement should be opened.

John Maddox

Trees: No news . . .

BRITAIN'S trees are reassuringly healthy, according to a report published by the UK Forestry Commission last week*. In a £40,000 survey during 1984, the commission found that no new phenomena were needed to explain unhealthy trees. Well-known pests, diseases and climatic influences accounted for all such cases. The survey was started because of increasing environmental damage to European forests, particularly in West Germany.

The main aims of the survey were first to assess major tree species to see whether the patterns of decline were similar to those in West Germany and elsewhere in Europe; and second, to relate the symptoms at particular sites to local conditions, especially with respect to atmospheric pollution. The species assessed in the UK survey were Norway spruce, Sitka spruce, and Scots pine.

The survey covered the whole of Britain, and included stands of 35–40-year-old trees likely to remain undisturbed for at least 10 years. Land was divided according to height, amount of rainfall and amount of sulphur deposition. (Sulphur dioxide was the only potential pollutant included in the survey; there is not enough information available on the distribution of rainfall acidity and ozone, for example, to include them in the study.) The Forestry Commission's subcompartment database was used to combine these data with, for example,

Espionage

Soviet scientist accused

A SOVIET scientist, employed at the Arctic and Antarctic Institute of the State Hydrometeorological Commission, made use of research trips abroad to pass on classified information to United States intelligence, according to *Pravda*. Soviet newspapers are usually reticent about reporting criminal or espionage cases, but this particular story was considered worthy of a three-part serial. During the past two years, the Party *agitprop* literature has paid considerable attention to the risks faced by Soviet sailors and others obliged to travel to the capitalist world. The history of the scientist-spy, Yuri V. Pavlov, (code-name Rolf Daniel) was presumably given such extensive coverage to show that even a highly educated Soviet scientist is not immune to temptation.

Pavlov, according to his own statement, quoted in *Pravda*, was the son of a mining engineer and an English teacher. Born in 1935, he studied in Leningrad, specializing in experimental nuclear physics, but abandoned his dissertation work out of boredom. After being involved for some time in a "fantastic project" to create artificial diamonds using nuclear explosions, he drifted out to the Soviet Far East and then, in 1961, took part in an expedi-

tion to Japan, Tahiti, Fiji and Hawaii aboard the doyen of Soviet research vessels, *Vityaz*. Here, *Pravda* implied, Pavlov proved a misfit, being temperamentally unsuited to carry out serious scientific work without "fanfares or laurels".

He returned to Leningrad and for the next seven years he apparently drifted from job to job seeking good pay rather than interesting work.

For a time, he was employed in what was entered in his official work record book as "secret work and documents". In 1969, he went to work for the Marine Register of the USSR and, in his own words, became "one of the central figures in the atomic inspection of the register". Between 1977 and 1980 he travelled abroad to meetings of an international working party on safety regulations for nuclear ships, visiting London (four times), Genoa, Ottawa and Hamburg. Finally he left this job however, feeling that it was "too narrow" for him and in the autumn of 1981 began working at the Arctic and Antarctic Institute, in order that he might avail himself of the opportunity of foreign travel aboard the research ship *Professor Vize*.

During his travels, Pavlov stated, he was recruited by West German intelligence and the Central Intelligence Agency. The *Pravda* narrative traces out the course of his contacts in foreign ports, the "spy kit" issued to him, the efforts of Soviet counter-intelligence to track him down and his final confession and repentance and the imposition of an (unspecified) appropriately severe sentence" (presumably the death penalty) by a military court.

The exact data which Pavlov allegedly handed over to the West are left vague, but a tailpiece entitled "instead of an epilogue" puts the story firmly in the context of US/NATO "aggression" as revealed by "star wars", psychological warfare and disinformation.

Ceaseless vigilance, says *Pravda*, is the "principal weapon" in this ideological war. Pavlov, on the contrary, exhibited the "lightmindedness" of "ideologically unstable people", who "live in a vacuum" and who must be excised from Soviet society as a "foreign body".

Perhaps the most telling summing-up of Pavlov's character faults, however, comes not in this tailpiece but in the body of the narration, where he is accused of "Manilovism". This word, drawn from the name of a character in Gogol's *Dead Souls*, does, indeed, typify the shiftlessness and lack of stability which, presumably, Pavlov exhibited, but it is a word that has not recently been much in vogue in Soviet character-analysis, having been one of Stalin's favourite epithets of condemnation.

Vera Rich

Nuclear waste

EPA regulates at last

Washington

THE US Environmental Protection Agency (EPA) has at last issued regulations that it wants to see enforced at high-level radioactive waste disposal sites. But the "final" standards are unlikely to be the end of a long inter-agency dispute on how the regulations will be enforced, and there seem likely to be many opportunities yet for opponents of geological disposal to mount legal challenges.

EPA has come out on the wrong side of an argument with the Nuclear Regulatory Commission (NRC) over regulation of disposal sites, the upshot of which is that EPA can only implore NRC to comply with a list of assurance requirements designed to ensure that there will be no significant leakage of radioactivity for 10,000 years after disposal operations are completed. The practices at the disposal sites while they are still in operation will be regulated by NRC, whose standards are different from those promulgated by EPA.

The need for a permanent resting place for US nuclear waste has become urgent in recent years. In 1982, a report from the Congress's Office of Technology Assessment warned that some power stations might have to close down unless provision was made for disposing of high-level waste, mostly used fuel rods, now accumulating in ponds at power station sites. Given the tarnished public image that nuclear power already suffers in the United States, that was an option to be avoided at all costs.

Responsibility for selecting and operating a disposal site rests with the Department of Energy, which is now engaged in processing some 20,000 comments on its proposal to carry out on-site investigations at three possible sites for a high-level waste repository. By the end of the year, the department hopes to have confirmed its previous choices and to have issued final environmental assessments. A final decision will then be made by the President and Congress.

The standards that EPA has now issued limit exposures to members of the public to 25 millirems to the whole body, 75 millirems to the thyroid and 25 millirems to any other organ. There is also a waiver whereby the EPA administrator can raise the 25 millirems limit to 100 millirems if he sees fit or 500 millirems for a one-off dose. And the standards limit projected releases of radioactivity to the environment for 10,000 years; EPA estimates that compliance with the containment requirements means that there will be no more than 1,000 cancer deaths over the entire 10,000 years considered. Tim Beardsley

... is good news

tree species and age, to provide the basis of stratified sampling schemes.

No needle yellowing, such as that found in Norway spruce in West Germany, occurred in Britain. A strong regional effect for crown density and needle age could have been caused by local environmental factors or by bias among surveying teams. Scots pine and Norway spruce hold their needles for longer at higher altitudes but had less dense crowns where rainfall is higher. Scots pine held its needles for longer, but Sitka spruce had less dense crowns, in high sulphur deposition zones. None of these signs of sickness was caused by previously unknown phenomena.

Although the news is good for Britain's trees, optimism should only be cautious. A single survey has limited use in detecting changes in forest health, but patterns should emerge with subsequent re-surveys. West German surveys cannot easily be compared with the UK data because of differences in management, altitude, climate and, particularly, age of trees (old trees are more susceptible to pollution damage). As the trees in the UK survey grow older and the local levels of pollution vary with time, patterns of damage, if they exist, will emerge.

Maxine Clarke

*Forestry Commission Research and Development Paper 142 Forest Health and Air Pollution: 1984 Survey £1.40.

Nuclear India

New reactor "not for bombs"

New Delhi

INDIA has denied reports that the country's largest research reactor, commissioned on 8 August, is intended to breed plutonium for bombs to counter Pakistan's nuclear threat. A spokesman for the Department of Atomic Energy (DAE) says that the 100-MW DHRUVA reactor at Bombay is capable of producing annually 30 kg of plutonium which will be outside international safeguards, "but it is not a dedicated facility". He says that like all uranium-fuelled reactors in the world, DHRUVA will breed plutonium, but is not linked with any weapons programme.

India is not in fact dependent solely on DHRUVA for plutonium if it did decide to go nuclear. It has been reprocessing spent fuel from CIRUS, an ageing Canadian-built 40-MW reactor operating in Bombay, since 1963. CIRUS was the source of plutonium for the nuclear test in Pokran in 1974. Another 100 kg of plutonium, also from CIRUS, was used to make the Pu-U carbide fuel for the Fast Breeder Test Reactor (FBTR) which is nearing completion at Kalpakkam near Madras.

Neither CIRUS nor the first 230-MW unit of the Madras Atomic Power Plant (MAPP-I) which has been operating since 1983 is under safeguards; these apply to the US-built power plant in Tarapur, and the two atomic power reactors in Kota, Rajasthan. While Tarapur fuel has not yet been reprocessed, every gram of plutonium produced at Kota has been accounted for. India's current stock of plutonium is estimated to be at least 200 kg. DAE officials point out that it is not from lack of plutonium but by a political decision that India has not been making bombs.

Four days after DHRUVA became critical, DAE commissioned MAPP-II, the second power plant in Madras. This "90 per cent indigenous" power plant, together with DHRUVA, which will replace CIRUS in five years, will no doubt add to the stockpile of unsafeguarded plutonium which DAE says is required to fuel the fast breeder reactors India plans to build in the 1990s. The FBTR, similar to France's RHAPSODIE, is expected to become critical in September and India is well advanced in the design of a 500-MW prototype commercial breeder.

According to DAE, the DHRUVA reactor will be used to produce isotopes for agriculture and medicine, besides research on solid state which requires a higher flux of neutrons than is available at CIRUS or APSARA—a one MW swimming pool reactor built in 1956 using British supplied enriched uranium. DHRUVA, which uses natural uranium fuel and a heavy water moderator, is designed for a flux of 10^{14} neutrons at the centre of the

core, and is thus comparable to the NRX reactor of Canada. DHRUVA will, however, operate at full power only after DAE has solved the serious problem of vibration of fuel rods and ensured complete safety of the interlock systems. When one of the interlocks failed two days before the reactor went critical, four ton-

The DHRUVA (left) and CIRUS (right) nuclear reactors.



Soviet alcoholism

Drunk driver stripped of degree

THE Soviet Union's new campaign against alcohol abuse has cost at least one scientist his degree, and the lifelong financial benefits that go with it. The legal provisions, introduced in 1976, allowing those found guilty of conduct unbecoming to a Soviet citizen to be deprived of their degrees, has so far been used only in a handful of cases involving Jewish refusniks.

The penalty has now been imposed in the bizarre case of Boris Samarin, a former employee of Leningrad University, who is just completing a four-year sentence for causing the deaths of six companions while driving under the influence of drink.

The accident, which took place in the small Karelian village of Suoyarvi, involved members of the Leningrad University Karelian Geophysical Expedition, headed by Samarin. On the way back from the expedition, Samarin was travelling in the cabin of the expedition truck, and both he and the driver, Viktor Kolesnik, were drinking heavily. When the truck ran off the road into a swamp, all six people in the body of the truck, three girl students, two women scientists and the small son of one of the latter, were drowned, Samarin and Kolesnik escaped to face the legal consequences.

Court cases are normally reported in the Soviet press only to illustrate some facet of current policy, and it was only last August, more than three years after the

crash, that the Moscow *Literaturnaya Gazeta* carried an extensive article on the incident, pointing out that Samarin was well known to be a heavy drinker, that members of the hydrogeology department were reluctant to go on expeditions with him (indeed, his expeditions regularly included a number of "dead souls", who figured in the official report but who had never actually left Leningrad), and that, nevertheless, no apparent efforts were made to curb his drunkenness nor to take the organization and running of expeditions out of his hands. How, asked the *Literaturnaya Gazeta*, could such a situation arise?

K.S. Jayaraman

A few weeks later, the newspaper printed a reply from the rector and the secretary of the Party Committee of Leningrad University. This, in effect, pointed out that the unfortunate incident had taken place three years previously, and that as a result of the tragedy, procedures relating to university personnel and to the organization of expeditions had been considerably revised.

Last month, as a postscript to the whole affair, the paper noted that it had received a letter from Dr G.M. Nesmeyanov, the head of the Higher Attestation Committee, VAK, the body responsible for higher degrees, saying that Samarin had now been deprived of the degree of Candidate of Geological and Mineralogical Sciences.

Vera Rich

Environmental organizations

Workings of Greenpeace

THE boats of Greenpeace both symbolize and implement the direct action campaigns by which that group enters some of the world's most highly publicized environmental controversies. Greenpeace's willingness to establish a physical if non-violent presence gives it an "extreme but not extremist" character. "People want to see effective environmental campaigning for their money", says one spokesperson, pointing to press coverage of the group's activities as one measure of fulfilment of that promise.



Although environmental groups sometimes formally pool their resources, such as in the consortium fighting the US sanctioning policy on Japanese whaling, the strength of the environmental movement, according to Peter Wilkinson, member of Greenpeace's international board, lies in its diversity. Greenpeace takes a radical stance amid this diversity, though not as radical as that of groups espousing violence. Alan Pickover, Greenpeace international coordinator for marine animal campaigns, says that the group's position on the outer fringes often serves to push governmental compromises towards the middle ground of environmental opinion, which would not happen if conservative groups such as the Royal Society for the Protection of Birds were the primary environmental voice.

The sinking of *Rainbow Warrior* has not changed Greenpeace campaign priorities, although the loss of the £250,000 investment has resulted in a scramble for campaign funds and rearranged schedules for other ships. Greenpeace offices worldwide, says Mr Wilkinson, have been inundated with messages of support, although the level of contributions has not yet been assessed.

With the loss of *Rainbow Warrior*, which was based in the United States, Greenpeace is left with only two large ships. The older of the two, *Serius*, has just left an aborted attempt in Iceland to stop that country from killing 200 whales next year for "scientific research". Only the first instalment has been paid on the ocean-going tug *Greenpeace*, now on its way to Mururoa and Antarctica. Green-

peace had bought this vessel before *Rainbow Warrior* was sunk and will have to pay the remaining \$360,000 over the next three years. The organization also owns a river-going vessel bought with publicly raised funds and equipped to detect pollution as well as a hot air balloon held by the East Germans ever since it inadvertently sailed over the Berlin Wall.

Greenpeace has offices in 15 countries. For an office to have a vote on Greenpeace policy, it must not only be self-sufficient but also contribute funds to international campaigns. Some of the six fledgling offices have simply not had the time to mature; others, such as the French office, are important for political reasons even if they do not make much money. Every fall, representatives from these 15 countries meet to discuss policy and international campaign budget issues. Their decisions determine the organization's priorities for the coming year as well as setting the official Greenpeace viewpoint on any given issue. An elected international board of five is responsible for monitoring as well as for emergency decisions in the course of the year.

Greenpeace offices worldwide are organized in different ways depending on individual countries' needs and laws. Only in the United States does Greenpeace maintain full-time government lobbyists because policy-making there is amenable to such pressure tactics. In Europe, on the other hand, lobbyists concentrate on international conferences. Each office supports at least one expert in each of the primary areas of Greenpeace activity, but campaign strategy in any area is settled at the international rather than the national level.

AIDS

US company rejects UK decision

THE UK Public Health Service (PHS) evaluation of test kits that detect the presence of antibodies to the AIDS (acquired immune deficiency syndrome) virus in the blood has been criticized by one of the companies that did not come out well from the study (see *Nature* 8 August, p.474). Of the five companies whose kits were evaluated, Wellcome Diagnostics (UK) and Organon Teknika Ltd (The Netherlands) were selected for the second stage of the evaluation, testing by the blood transfusion service. By October, all blood donated in the United Kingdom will be screened for the presence of the AIDS virus, probably using one of these kits.

But Abbott Laboratories Ltd, US manufacturers of one of the unsuccessful kits, is not satisfied with the results of the PHS evaluation. The company is lobbying government officials and physicians in the

International campaign proposals fall into four categories: nuclear; marine animals, which includes fisheries; chemicals; and terrestrial animals. Before the annual meeting, the campaign coordinators responsible for these areas, armed with figures of the available funds, work out proposed budgets with the campaign directors in each area. Greenpeace commissions technical reports and also uses experts on each animal or topic at issue to determine where the greatest needs lie. The compromise decisions that are made at the annual meeting tailor the proposals to stay within the expected income for the coming year. In 1985, 29.1 per cent of direct action money was spent on marine animals, 22 per cent on nuclear, 12.4 per cent on chemicals and 1.1 per cent on terrestrial animals (mostly kangaroo pelts and meat). The high cost of the boats eats up most of the remaining 35.4 per cent; a three to four-day action in the North Sea, for example, costs roughly \$20,000.

Trouble can arise because of unexpected losses such as *Rainbow Warrior* or if offices do not raise the "notional money" they had pledged and which was divided up in the annual meeting setting out goals for that year. After the budgets for running national offices are deducted, the remaining funds from all the offices are pooled to cover Greenpeace's international campaigns. The estimated income for 1986 is \$10 million, with 80 per cent of that available for campaigns. According to one spokesperson, Greenpeace income has been increasing at roughly 10 per cent a year, with the average donation \$10-100 and virtually no donation over \$1,000. There is no extra room in the budget. According to Mr Wilkinson, Greenpeace's bank balance after campaigns is only \$300,000, which represents a dangerously small margin of leeway.

Elizabeth Collins

United Kingdom to persuade them that the results of the survey are "preliminary and misleading". Kits currently available on the US market, which include Abbott's, are almost completely efficient at detecting AIDS virus antibodies.

Whether Abbott's complaint is justified will not be known until PHS publishes the details of its evaluation early next month. If Abbott's test is not one of those recommended by the health department to the health authorities for use in the transfusion service, the company will lose out in a business that is worth £5-10 million a year. Abbott claim to have a prototype kit that detects viral antigen, a test which would enable much earlier identification of people at risk to or carrying the AIDS virus and which is recognized to be the next step in the control of the disease.

Maxine Clarke

Animal intelligence

SIR—According to Geoffrey Hall's review of *Animal Intelligence* (*Nature* 25 July, p.306) "... modern experimental studies of animal learning... have found precious few differences between species that can be unambiguously interpreted as being the result of differences in intelligence. Macphail concludes that we might as well accept... that extant non-human vertebrates do not in fact differ in intelligence". I would like to support the reviewer's comment that this conclusion is a condemnation of the skills of modern experimenters rather than an accurate judgement of the intelligence of animals. There is surely something very wrong with studies that show no unambiguous differences in intelligence between a frog or a chameleon — or even a sheep — and a sheepdog or a chimpanzee.

Very complex behaviour patterns can be built into the simple nervous system of an insect; a honey-bee can quickly learn to find its nest by what must be a quite comprehensive imprinting essential to the success of its way of life. The ability of a rat to learn its way through a maze may not have much relevance to its intelligence. Young rats may be programmed to learn complex and growing mazes of tunnels.

The primary function of intelligence must have been to improve the chance of survival or it would never have evolved. For a specific way of life in an unchanging environment, a built-in behaviour pattern that has been optimized by natural selection over 5 million years — a typical species lifetime — is not easily improved by an individual creature. Improvement must require occasional replacement of part of the built-in behaviour pattern by an anomalous behaviour locally more successful than the five-million-year optimum. This in turn requires three things. The first is a good memory of events in the individual's life, such as stumbling on a novel source of food in an accidental excursion into a normally avoided environment, or of a narrow escape by novel means from a novel hazard. The second is the ability to recognize the value of repeating the experience. The third is the ability to override the built-in behaviour pattern in order to do so.

The enormous gap between human and even the greatest animal intelligence could mean that we ought to use different words for them, but I believe that the difference is quantitative rather than qualitative. We passed a vital threshold when we became bright enough to carry throwable chipped stone weapons. A group so armed would have become very unattractive to the big predators, and survival from then on must have depended on intra-specific competition for food-bearing territory between groups. This did nothing to make us tolerant of groups of strangers but a lot to increase our intelligence. A second

threshold was passed when we developed language. The value of this in original practical thinking is often exaggerated, but its value in transmission of thought and knowledge and hence in inter-group fighting was overwhelming.

The value of intelligence will be much increased if the environment undergoes a large and permanent change. At the end of the Cretaceous period, the Earth seems to have been struck by a large meteorite and the resulting dust clouds may have persisted long enough to eliminate many species of plants and animals over large areas. The survivors will then have been thrown suddenly into a changed biological environment in which many of the less adaptable species may later have died out. Then those species, for example the early mammals, that could diverge from their built-in behaviour, and learn from their own experience and from novel behaviour of parents or other conspecifics, would have had a great and sometimes overwhelming advantage over those with rigid behaviour patterns.

To measure intelligence we must do

more than measure memory. The ability of a rat to learn a maze is a good test of memory, but could be using the same kind of built-in learning pattern as that of the honey-bee. It would be better to give the rat a chance of some lateral thinking by, for example, painting a particular scent on the wall in the middle of each straight section on the side to which the rat should turn at the next fork. Increased speed of learning another maze with the same cues could hardly result from any built-in pattern. Similar problems could presumably be arranged for a goldfish, a tortoise, or more expensively a chimpanzee.

To summarize, I believe that there are differences in intelligence, which must in principle be observable, between different vertebrates, and that there are understandable reasons why these should have arisen. Furthermore, in testing for intelligence, I believe that one should try to distinguish between memory and the ability to notice correlations between experiences, and should look for ability to override normal or usual types of behaviour.

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PhD theses

SIR—A PhD is supposed to be a training in research. Normally this process is conducted over a period of three years, financed by either government or industry, and involves three years' supervision by a university teacher, to which must be added the cost of equipment both capital and consumable, and technical assistance over the requisite period. The return for the many thousands of pounds of outlay per candidate is a thesis on a university library shelf and a piece of paper for the successful candidate which hopefully will enable him or her to command a higher salary than he or she could otherwise have expected.

While it is true that theoretically theses can be consulted in university libraries, in practice this is rarely done. References to PhD theses occasionally appear in the scientific literature, but usually only when an author refers to his own unpublished thesis.

Research is not recognized as having been brought to a conclusion until it has appeared in print in a recognized scientific journal. An essential part of training in research should be the preparation of the work for publication as a scientific paper. A PhD should not be awarded until there is evidence that the major new contributions to science have been published or at least accepted for publication in an appropriate reputable journal. If the material is not suitable for publication then it was not necessary for it to be done in the first place. If there are a large amount of basic data for which there should be ready access, then this could be

deposited in suitable archives as is already the practice with learned societies.

It must surely be time for the antiquated PhD thesis system to be thoroughly overhauled. The stupendous waste of resources cannot be justified at this time. It cannot be beyond the wit of man to devise a system more in keeping with present needs.

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Modest disclaimer

SIR—John Maddox's readable account (*Nature* 11 July, p.101) of the experiment that T. Corle and I made to measure Maxwell's displacement current inside a capacitor as it is being charged does us too much credit. Although we may have been the first to measure the profile of the magnetic field between the capacitor plates, we were not the first to measure the total magnetic flux.

That experiment was done almost 60 years ago by M. R. van Cauwenberghe (*J. Phys. Radium* 10, 303; 1929). The title of that work, "Vérification Expérimentale de L'Équivalence Électromagnétique entre les Courants de Déplacement de Maxwell et les Courants de Conduction", aptly expresses the results of a very pretty measurement which used an iron-core, toroidally-wound solenoid to measure the magnetic flux through the central portion of a capacitor.

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Rates of galactic star formation

A remarkably simple argument successfully accounts for the rate of star formation in different galaxies. The snag is that the timescale is uncomfortably short.

How could star formation be infectious, or autocatalytic, "catching" in the vernacular? Simple prejudice says that such a mechanism is too anthropomorphic to be real. But the simple sight of a spiral galaxy such as that of the Milky Way will show that the pattern of star images, but especially that of new stars, has a clumsiness that suggests their congregation. The mass of stars in the Orion nebula argues the same case. The objective way out of this dilemma is to follow Schmidt (originator of the fast sky-camera) in supposing that the rate of star formation is somehow a function of the density of the raw material, interstellar gas. Obviously the functional relationship cannot be linear, for then the ratio of stellar mass to that of the immediately surrounding gas would be uniform in space. Schmidt (*Ap. J.* **129**, 243; 1949) suggested that the rate of star formation might be proportional to the square of the density of interstellar gas, or something like that.

Michael A. Dopita, from the Mount Stromlo and Siding Spring Observatories, who has a better autocatalytic idea, points out (*Ap. J.* **295L**, 5; 1985) that no relationship of the type proposed by Schmidt can explain why the Smaller Magellanic Cloud has only one fifth as much mass locked up in stars, per unit mass of gas, as has the Larger Magellanic Cloud. Infection, autocatalysis or positive feedback in star formation is his solution.

Suggestions that star formation may stimulate further star formation are far from novel. The mechanics of such a process are easily described. Newly formed stars will heat and even ionize the immediately surrounding interstellar medium, while those massive enough to run through their evolution quickly to form supernovae will add hot material to their environment. The result is that the interstellar medium is filled with hot gas in a set of circumstances, far from equilibrium, in which the colder material in the environment is compressed into clumps that may be massive enough to collapse under their own weight into proto-stars and eventually stars. Dopita refers to several explicit accounts, going back to the late 1970s, of how such a mechanism may account for observed properties of galaxies.

His own contribution is what he calls a "law of star formation" to replace that originally suggested by Schmidt. Time will tell whether it provides a sufficient ex-

planation of the variation of the rate of star formation from one galaxy to another as well as from place to place within a single disk-shaped galaxy. But its immediate virtue is the simplicity of its argument.

To make sense, a law of star formation must link the observed rate of star formation with something else that can be observed. Dopita settles for the surface brightness of neutral hydrogen of a galaxy, or of a part of it, which the radioastronomers are able to measure directly. The essential link between these quantities is simply that of kinetic theory, that the mean squared velocity of the gas is proportional to the pressure.

Dopita's argument then starts from the assumption that the interstellar gas pressure is a linear function of the two quantities representing the mass density of stars and the rate at which that variable changes with time as a consequence of star formation. In gas-rich galaxies (such as both the Magellanic clouds) but also in other circumstances, the rate of star formation will be the dominant determinant of interstellar gas pressure. In those circumstances, Dopita concludes, the total rate of star formation in a galaxy should be proportional to the product of its total mass and the surface gas density (in grams per cm^2 , for example).

The prescription seems to work remarkably well. At least on a log-log plot (of the rate of star formation and the product of total mass and surface gas density), both the Magellanic clouds, our own galaxy and a score of other gas-rich galaxies lie within a factor of less than 10 of a straight line, which is probably as much as can be expected for quantities such as the rate of star formation whose measurement is more a matter of inference than of observation. Certainly the result will be taken as an explanation of why the Smaller Magellanic Cloud has many fewer stars than its companion satellite galaxy — its mass is smaller.

The more typical case in which the interstellar gas pressure is a function both of the rate of star formation and of the density of pre-existing stars leads to a more complicated but still simple relationship. One necessary step in the argument is to estimate the fraction of the material incorporated into new stars which remains locked up in them. They must be objects not so massive that they form supernova explosions. The result, however, is a first-order linear differential equation for the

surface density of gas as a function of time. Integration leads directly to an expression for the surface gas density as an exponentially decreasing function of time which, significantly, does not decrease to zero but, rather, to a non-zero value. In other words, even in the galaxies most productive of new stars, there will always remain a certain amount of gas that is not incorporated into stars.

Again, the agreement with observation is good. The surface density of gas as a fraction of the total surface density of a galaxy should be a straight-line linear function of the ratio of the star-formation rate to the total mass of stars, either for an entire galaxy or for part of it. In the sample of galaxies Dopita uses for the comparison, the only two galaxies that do not lie near the curve are, in a sense, exceptions that "prove the rule" — other evidence suggests that they have recently accreted gas from some extragalactic source, and are thus artificially young.

Dopita himself says it is remarkable that "such a simplistic" model should give such a good fit. He also points to what may be the most uncomfortable consequence of the argument, the short timescale for galactic evolution implied by the constants in the model, determined by the best fit of the observations with the predictions. Most galaxies, on this basis, are using up their gas content on a time scale which is short, on the order of 1,000 million years, compared with the time scale of the Universe. Dopita is again quick to point out that this difficulty has arisen previously, most notably in the work of the late B.M. Tinsley and her colleagues but specifically in work such as that of R.C. Kennicutt (see *Ap. J.* **272**, 54; 1983), in which ultraviolet emission from hydrogen is used as a direct index of star formation.

On the face of things, this implies that the history of the present era of star formation in isolated disk galaxies is shorter than has previously been assumed and that, for some galaxies, the renewal of the stellar population may have only a short way to go. The puzzle is an important conundrum whose resolution is nevertheless not entirely beyond the bounds of possibility. Plainly the Dopita law does not take account of all the possibilities. But any explanation can only further complicate the difficulty of learning enough about the evolution of galaxies to tackle the question of the evolution of the Universe as a whole.

John Maddox

Palaeoanthropology

Palaeobiology and age of African *Homo erectus*

from Eric Delson

FINDS of ever-older human relatives and potential ancestors have caught the public's imagination in recent years. Kenyan fossils newly described on page 788 of this issue by Brown *et al.*¹ could help to swing the pendulum of public interest back toward younger fossils belonging to species of our own genus, *Homo*. Since 1969, Richard Leakey and his colleagues have recovered human and other fossils from deposits along the eastern shore of Lake Turkana in northern Kenya. Now, a group has crossed to the west side of the lake; early work has yielded a fossil already touted in the press as a "strapping youth" whose bones will shed yet more light on human ancestry².

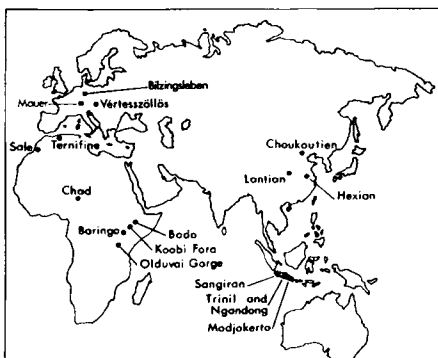
The new fossil has been identified as a representative of *Homo erectus*, the extinct species first named *Pithecanthropus erectus* or "Java Man" by Dubois in 1894. Skulls of many individuals of this form are now known from Java and China, mainly estimated to date between 1.2 and 0.25 Myr old^{3,4}. It is widely accepted that populations similar to *H. erectus* were directly ancestral to the earliest members of the living species *H. sapiens*, although the exact timing, geography and mode of transformation are still controversial. Unfortunately, limb bones are scarce in the Asian sites, and some of the most complete have been questioned as to their specific identification⁵.

That is the main value of the new Kenya fossil — it preserves almost all of the skeleton of a 1.6-Myr-old individual who can be sexed (as male, by pelvis shape) and aged (12 years, from tooth eruption timing). Fossils identified as *H. erectus* have been known in eastern Africa since 1960, when Louis Leakey reported a skull cap from Olduvai which matched those from Asia. Moreover, Olduvai and other sites have yielded partial limbs and pelvises attributed to *H. erectus*, but none has been associated with a well-preserved cranium, always rendering precise taxonomic identification doubtful. The West Turkana fossil (catalogued as KNM-WT 15000) preserves most of the skull and lacks only the extremities and forelimbs (the right humerus remains). The team expects to find additional portions of the skeleton with further excavation, but this degree of preservation is otherwise unknown before the Neanderthals of 40,000–100,000 years ago. By comparison, 'Lucy', the 3-Myr-old partial adult female skeleton of *Australopithecus afarensis* recovered by Johanson⁶, has a lower jaw but only bits of the skull; many fewer ribs and vertebrae; the upper and lower leg bones of only one

side of the body; but does preserve most of the two forearms and left humerus missing in WT 15000.

What will this new information tell us about how *H. erectus* lived? That is still hard to say, but one point much emphasized is the apparently great height of the individual: 1.6 m (5 ft 5 in) at death, perhaps 1.8 m (6 ft) if adult². Brown *et al.*¹ have moderated this claim, especially because the skull of *H. erectus* was lower than that of the modern *H. sapiens* whose bones were used to develop the height regressions; the regression estimates should consequently be reduced.

Previous estimates for the height of *H. erectus* ranged between 1.55 and nearly 1.8 m^{7,8}. They were usually based only on the length of the femur (thigh bone) and



Locations of major *Homo erectus* sites.

There is controversy over whether the European and Baringo fossils should be allocated to *H. erectus* or *H. sapiens*¹².

did not consider skull height differences. Thus, the WT fossil might not have been especially large for this species to begin with. Moreover, additional cavils need to be evaluated. Modern humans show an adolescent growth spurt, especially in males. If such a spurt existed in *H. erectus*, it might have led to a height greater than the estimated 1.6 m, had the specimen lived longer.

Similarly, it is unclear that dental eruption timing then was the same as it is today; T. Bromage and C. Dean have questioned the equivalence of such timing in *Homo* and *Australopithecus* species based on counts of perikymata (developmental lines in tooth enamel)⁹. On the other hand, if most *H. erectus* individuals were rather shorter than WT 15000 (which is less likely), it is possible that this individual was atypically tall or died young because selection acted against his continued growth.

Palaeoanthropologists will probably be more interested, however, in the insights that this fossil will provide into the

patterns of growth and inter-individual differences among earlier humans. As evidenced by both dental eruption and epiphyseal fusion stages, WT 15000 was clearly juvenile. Yet Brown *et al.*¹ note that the face is larger and more massive than that of an adult *H. erectus* skull from the east side of Lake Turkana they have just described¹⁰. That skull, ER 3733, is widely accepted as female, so that its relative gracility compared with WT 15000 is not surprising (except for the age of the latter). It would be interesting to compare WT 15000 with ER 3883 (ref. 10). Some authors think the latter was a male (ref. 8) but I consider it another female and the large but stratigraphically younger Olduvai OH9 to be the male in this sexually dimorphic species.

This question leads inexorably to the two main recent foci of attention on *H. erectus*: does the species illustrate stasis or phyletic change through its 1.5 Myr span; and are the African forms really the same species as the Asian? One of the most important types of information that bear on both these questions is accurate ages for the fossils. Although Pope³ and other workers have recently argued that the oldest Asian *H. erectus* are often significantly younger than previously thought (1–1.2 Myr at most), careful re-analysis of several East African geological sequences has clarified the age of the earliest supposed members of the taxon there. Also in this issue, McDougall *et al.*¹¹ on page 792 present new data on the age of one major Turkana Basin marker horizon, the Okote Tuff complex. They report that the basal levels of this 10–15-m-thick unit date to no more than 1.64 Myr. The pumice fragments dated have a composition different from that of the enclosing tuff, suggesting reworking upwards of older pumice into the slightly younger tuff. Brown and Feibel¹² on page 794 offer further local correlations and review other dates which indicate that it is not much younger (>1.52, probably >1.6 Myr). In addition, they determined that a tuff slightly below the horizon of WT 15000 is equivalent to one of two newly defined tuffs on either side of the Lower Okote, yielding the age of 1.55–1.65 Myr for the skeleton.

These dates and others in the Early Pleistocene (through to perhaps 1.0 Myr) make it simple to begin the study of temporal variation in *H. erectus*. But as noted by Pope³, dates in Asia are still highly uncertain and younger African fossils (especially in the Maghreb) are also of questionable age. Thus, although studies such as those of Howells¹³ and Rightmire¹⁴ have suggested that variation through time is not continuous or even directional in *H. erectus*, Wolpoff¹⁵ has recently argued strongly for a gradualistic view of temporal variation and change in this species. His comparisons of 13 cranial characters across specimens grouped into three time ranges are superficially persuasive, but there are too many potential problems

for his analysis to be convincing. Several individual fossils are of questionable identification, sexual variation is not considered and the raw data are not available to see the precise effects of these factors. Equivocal age determinations and the use of time bands rather than age estimates that would permit regression analysis also disturb me. As Wolpoff notes, it has been "excruciatingly difficult to test the punctuated equilibrium model in the fossil record", not least because of methodological disagreements among researchers.

An examination of Wolpoff's list of specimens reveals that whereas East African and Indonesian fossils dominated the 'early' and 'middle' *H. erectus* time groups, the sample from Zhoukoudian, China dominates the 'late'. Some authors have recently questioned whether such comparisons are as meaningful as we might like—are the African fossils indeed members of a palaeobiological species *H. erectus* as defined from the unique characteristics of the Asian specimens defining this taxon? Howell¹⁶ reviewed the consensus position of geographical identity some years ago, providing a detailed descriptive definition without specific regard for the derived versus the conservative nature of the characters discussed. Rightmire^{17,18} has been the most vocal recent proponent of this view, arguing that several uniquely derived characters define *H. erectus* in Asia and Africa without recourse to the arbitrary use of temporal gaps or rubrics.

On the other hand, Wood¹⁹ and Andrews²⁰ argue strongly that African fossils assigned to *H. erectus* share only conservatively retained features with Asian members of that species, not any of their derived characters. Thus, they imply the need for recognition of a new species to receive African fossils possibly intermediate between *H. habilis* and *H. sapiens*. Stringer²¹ is more cautious, but agrees with

the lack of shared derived features in specimens from the two regions; he notes further that if *H. erectus* were to be defined broadly enough to include all of these fossils, most derived conditions seen in the younger Asian specimens (especially those found in China) would reject the concept of stasis in favour of definite change over time.

Palaeoanthropologists continually make the claim that more fossils will help answer pressing questions, and field workers who find them reap well-deserved rewards. If WT 15000 is any example of the quality of

material to be expected in future years from the West Turkana sequence, there will certainly be much grist for our mills. But the milling process must include careful and probing analyses of all the available fossils, matched with a willingness to test both revisionist and entrenched hypotheses and to embrace new interpretations, if we are to take full advantage of the hard-won fossils. □

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Genetic diseases

Probe for muscular dystrophy

from P. Michael Conneally

DUCHENNE muscular dystrophy (DMD) is probably the most common X-linked disorder in man, affecting one in 4,000 males, the disease being transmitted by carrier females. Sisters of affected boys have a 50 per cent risk of being carriers, but there has been no reliable test for prenatal diagnosis or for carrier females, although serum creatine kinase levels are elevated in approximately 70 per cent of carriers¹. The locus for DMD is in the middle of the short arm of the X chromosome (Xp21) and two DNA probes that flank the locus have been reported within the last two years², but unfortunately they are quite far away. Elsewhere in this issue Monaco *et al.*³ describe the successful cloning of a DNA fragment that detects a deletion which is in or very near the DMD gene. This is a major breakthrough that forms the basis for much more accurate detection of females carrying the gene and for prenatal diagnosis, and that will also help us to an understanding of the aetiology of this lethal disease.

About 600 males affected by DMD are born annually in the United States alone. It is a progressive muscle-wasting disease with onset before the fifth birthday; victims are confined to wheelchairs by their early teens and usually die before they reach their early twenties. In families with one affected boy, 50 per cent of his sisters are likely to carry the gene, so about the time the affected boy dies, his sister may be giving birth to another affected boy. The new probes will mean that accurate prenatal diagnosis and carrier detection will now be available for families with affected children, and so should lead to a substantial decrease in the burden the disease places both on families and health services.

The method that Monaco *et al.* have used, which is reported in detail in a separate publication⁴, involves a very clever technique that can also be used for a number of other genetic disorders. The method allows the cloning of DNA missing from patients homozygous (or

hemizygous in the case of X-linked traits) for a chromosomal deletion. The principal of the technique is to hybridize excess DNA from the patient with the deletion to DNA from normal humans: the DNA that fails to hybridize is consequently enriched with sequences from the region of the deletion. As an extra precaution, in the case reported here, the DNA from a boy with DMD (and other problems, like retinitis pigmentosa) and a small deletion in the short arm of his X chromosome was hybridized to DNA from cells with an excess (five) of normal X chromosomes. This method is likely to be generally useful in cloning DNA containing the affected gene in specific genetic disorders involving chromosome deletions or translocations. In the past few years, it has been possible to go from genomic DNA libraries to chromosome-specific libraries and in many cases now to site-specific probes. The approach of cloning DNA from chromosomal deletions should accelerate the tedious task of finding the gene responsible for a genetic disorder, as it greatly narrows down the set of sequences from which the gene has to be isolated.

Now, Monaco *et al.*³ describe how seven of these probes from the region deleted in one DMD patient were used to check for deletions in other DMD patients and also to do linkage analyses. Five of 57 males with DMD were found to have deletions, each missing at least 38 kilobases of genomic DNA. Two subclones detect restriction fragment length polymorphisms, which can be used to identify particular regions of DNA (for an explanation of this technique, see ref. 5). These subclones were used in a linkage study, showing that the clones represent DNA very closely linked to the DMD locus, so they can be used as a marker for the gene. It is interesting that nine per cent of the mutations at the DMD locus are deletions. In two other X-linked disorders, Lesch-Nyhan syndrome and ornithine transcarbamylase deficiency, deletions have been found in 18 and 7% of cases respectively^{6,7}.

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DMD is a genetic lethal because affected males do not live to reproduce. As the frequency of the gene will be twice as high in females as in males, one-third of the DMD genes are lost each generation. A similar number of new mutations arise to take their place. Thus, if male and female mutation rates are equal, one third of all DMD cases should be new mutations. There is quite a lot of controversy over this estimate but these probes should now enable us to resolve the dispute, as well as determine the relative mutation rates in the sexes. The most exciting aspect of these findings is that in the very near

future the DMD gene should be cloned and transgenic mice produced. These are major steps in determining the aetiology of DMD, which will in turn lead to fundamental insights into the basic mechanisms of this neuromuscular disease. □

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Immunology

Are peptides good antigens?

from G. Ada and J.J. Skehel

IT is generally considered that most antibodies elicited after immunization with a protein in its native conformation will react well with the native molecule, but that only a minority will react with the denatured protein, with linear peptides derived from it or with synthetic peptide analogues. Initially, these conclusions came from studies (reviewed by M.J. Crumpton, ICRF) of the antigenicity of proteins such as myoglobin and of tobacco mosaic virus (TMV), in which it was also observed that antibodies to modified proteins on binding to native molecules caused changes in their confirmation—for example, the binding of anti-apomyoglobin to myoglobin resulted in the release of the haem prosthetic group. Similarly, when protein fragments or synthetic peptides were used as immunogens, the resulting antibodies reacted poorly with the parent molecules. In the past few years the use of synthetic peptides as antigens has been extensively documented, and a Ciba Foundation Symposium was recently convened* to discuss advances in our understanding of the antigenicity of synthetic peptides and of their usefulness as biological reagents and as components of future vaccines.

Evidence in favour of the importance of segmental mobility of TMV protein in antibody recognition was reviewed by A. Klug (MRC, Cambridge). Seven continuous epitopes in the TMV protein have been located to regions in the molecule which correspond to sequences with high local mobility, as shown by X-ray crystallography. Such observations suggest that the local mobility of the polypeptide chain facilitate interaction of antibodies with the protein antigen, and that antibodies to these regions of high mobility react with peptide analogue because the peptides in solution can assume the conformation of the mobile segments. If, however,

peptides longer than eight residues are used to assign regions of antigenicity, additional epitopes may be found, corresponding to more structured regions of the molecule, including α -helices.

The molecular basis of the antigenicity of synthetic peptides was also considered by R.A. Lerner (Scripps Clinic) in a nuclear magnetic resonance study of the conformation of a peptide from influenza haemagglutinin. In solution the nine-residue peptide was found to contain a type II β -turn stabilized by a hydrogen bond between the carbonyl of the first residue and the amide group of the fourth, a conformation different from that which the peptide assumes in the native molecule. The possible antigenic significance of such a structure may be revealed by analysis of the structure of peptide-antibody complexes that are available.

As a contribution to the study of the degree of protein flexibility involved in antigen-antibody reactions, A.B. Edmundson (University of Utah) reported a study by X-ray crystallography of the interaction between a number of synthetic peptides and the binding site in an immunoglobulin light-chain dimer. N-formylated peptide antigens assumed shapes which conformed to the space available for binding in the main cavity of the site. The conformation of the binding site also changed to improve structural complementarity with the peptide ligand. Among the adjustments noted were rotations and translations of aromatic side-chains and expansion of the binding cavity by movement of the first, second and third hypervariable loops.

Several contributions dealt with practical aspects of the production of anti-peptide antibodies and peptide synthesis. PPD (purified protein derivative of tuberculin) reacts well with T cells but poorly with B cells. Coupling PPD to peptides enhances anti-peptide antibody production in animals pre-sensitized with BCG (P.J. Lachmann, MRC, Cambridge).

When inoculated into BCG-sensitized rats, PPD-peptide conjugates cause delayed-type hypersensitivity reactions which facilitate retention of the complex at the site of injection and possibly contribute to enhanced antibody production. M. Sela (Weizmann Institute) reported that covalent attachment of muramyl dipeptide to a synthetic poly-DL-alanine-diphtheria toxoid octapeptide conjugate greatly increases its immunogenicity. Immunity to cholera toxin can be elicited either by a polymerized peptide from the toxin or by peptide conjugated to a lipid moiety such as the dipalmityl lysine group. M. Geysen (Commonwealth Serum Laboratories) summarized a procedure for the rapid synthesis on a solid support of a large number of peptides and its application in a study of a putative discontinuous epitope of foot-and-mouth disease virus, the main antigen determinants of which are on the VP1 protein. A monoclonal antibody against the epitope which neutralizes virus infectivity but does not react with isolated VP1 polypeptides or with synthetic hexapeptides covering the complete VP1 amino-acid sequence was used as template. Peptides of increasing length were synthesized and assayed for antibody binding after addition of each residue, some of which were β -alanines to allow structural flexibility. Finally, an octapeptide that bound to the antibody to high titre was obtained.

Two papers were presented on the antigenicity of peptides corresponding to the repeat sequences which occur in many malaria antigens. V. Nussenzweig (New York University Medical Center) described studies of the species-specific circumsporozoite protein (CSP) in which a single epitope has been identified for all monoclonal antibodies. A dodecapeptide containing three four-residue repeats conjugated to diphtheria toxoid induces the formation of antibodies which react with both CSP and the immunizing peptide and which, in an *in vitro* system, is 90 per cent effective in preventing infection. Such preparations are expected to form the basis of an antimalarial vaccine. R.F. Anders (Walter and Eliza Hall Institute) reported on two antigens, S and RESA, isolated from the merozoite stage of *Plasmodium* infections. Peptides equivalent to portions of the repeat sequences of the S-antigen, which is released on schizont rupture, have been used to show that anti-S antibodies present in sera from infected individuals are specific for the repeat sequences. At present, such antibodies are not implicated in protection from malaria infection. RESA, the ring erythrocyte surface antigen, is inserted into the erythrocyte membrane at the time of merozoite invasion. It contains blocks of related but different repeat sequences; peptides equivalent to these repeats elicit cross-reacting and repeat-specific antibodies. The biological significance of these findings was discussed, particularly

* 'Synthetic Peptides as Antigens', London, 4-6 June 1985. The proceedings will be published under the title *Synthetic Peptides as Antigens* by Pitman Publishing, London, in February 1986.

in relation to the possibility that such cross-reacting antigens produced at different stages in the life cycle of the parasite may modify the host immune response to the parasite's advantage.

V.C. Stevens (Ohio State University) reported the use of a 37-amino-acid synthetic peptide corresponding to the carboxy-terminus of the human placental hormone, chorionic gonadotropin (hCG), as a component of a birth control vaccine. Since this sequence is not shared by pituitary hormones of similar structure, its use as an iso-immunogen is considered acceptable. The length of the peptide, which appears to contain type II β -turns in solution, is critical for optimum inhibition of biological activity. When conjugated to diphtheria toxoid and administered to female baboons in a squalene-water emulsion with muramyl dipeptide, the peptide prevents 95 per cent of pregnancies; in due course fertility is regained.

Two presentations provided examples of the use of anti-peptide antibodies as experimental reagents. In an analysis of the site on human IgG responsible for the production of auto-antibodies (rheumatoid factor), a peptide analogous to the glycosylated region in the γ 1-domain has been found to be the most active in eliciting the autoimmune response (D.R. Stanworth, Birmingham University). Differences in the extent of glycosylation at this site may contribute to its autorecognition. Experiments were also described on the use of ϵ -chain peptides to elicit antibodies which might modulate IgE-mediated hypersensitivity in rats and humans. G. Evan (Ludwig Institute, Cambridge) described the use of peptides to produce monoclonal and polyclonal anti-

bodies against the product of the *myc* gene and their use to identify the intact *c-myc* phosphoprotein in normal and transformed cells. They have also been used to localize the protein in cell nuclei in culture and in tumour masses by whole-body detection of ^{131}I -labelled antibodies.

Considerable enthusiasm has been generated for the use of peptides as components of vaccines, and the results obtained with CG and malarial CSP peptide analogues have gone some way to support this. In both cases, the size of the immunizing peptide is important and this may indicate a requirement for more than one sequence in a peptide to be recognized, or that peptides of these sizes more readily assume conformations required for immunogenicity. But it also became clear that many synthetic peptides do not induce antibodies that react with the native parent protein, arguing against the general applicability of peptides in vaccine production at this stage. Such observations also have implications for the interpretation of basic studies of protein antigenicity in which the definition of antigenicity is simply based on the characteristics of the assay systems employed, which often involve the use of peptides or modified proteins as antigens. As a consequence the criteria which have been singly deduced from these studies to be indicative of antigenic regions in native proteins are unlikely to be universally applicable. $\square$

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Computers

More parallel than others

from D. Parkinson

PARALLELISM in computers takes many forms. Single operations use parallelism to enhance performance by, for example, fetching all 'bits' of a word simultaneously from memory or by using parallel circuitry to speed up operations such as addition or subtraction. Single-operation (bit-parallel) has been common since the earliest computers, when manufacturers began to use 'pipelined' operations to speed up the execution of streams of instructions. Pipelining is the computer's equivalent of the industrial production line, in which a complex operation is split into several sub-tasks and the operands are fed through a series of specialized facilities that perform these sub-tasks. It is a well-established procedure in computer design and usage, but the invention of the vector processor and creation of general purpose multiple-processor systems have awakened new interest in the possibilities

of parallel processing.

As a trivial example, imagine the following basic operations on a stream of instructions: fetch the instruction from memory; decode the instruction; obey the instruction. A three-stage pipeline could be constructed that would allow all three instructions to be analysed simultaneously. The first would be obeyed while the next is being decoded and the third is being fetched, the process being three time faster than a non-pipelined system.

The pipelining principle is also well-established for building high-performance signal or process-control systems. Here the tasks in the pipeline might be allocated to separate special-purpose boxes for performing operations such as calibration and convolution or transforms. Both these applications of pipelining are examples of parallelism, but they display parallelism at different levels of 'granularity'. The

instruction-decoding example is fine-grain parallelism where the amount of work between synchronization activities is small. The signal processing application exploits coarse-grain parallelism where large amounts of computation are carried out between synchronizations. There is, of course, nothing that prevents the operations of the coarse-grain system being implemented by systems that exploit fine-grain parallelism.

The vector processor, such as CRAY-1, CYBER-205, is a further refinement of the pipeline principle to perform repeated application of identical instructions to sets of data, the so-called vectors. If we wished to perform the operation $x(i) + z(i)$ ($i = 1 \dots N$), then increased performance is possible if the hardware can perform the instruction-decode operation only once and include all indexing operations in the pipeline. Vector processors have special facilities to perform these operations and so can provide increased performance for that range of computations which can be expressed in terms of the vector operations. The instruction-decoding base for a vector instruction is more complex than for a simple scalar instruction, so there must be minimum number of elements in the vectors before vector instructions can be faster than a set of scalar instructions. This characteristic of vector processors means that the programming or computational methods for full exploitation of a vector processor can be very different from those used in the simple early computers.

Alongside the development of vector processors, multiple-processor systems have been developed consisting of many interconnected computers, all of which can be used to solve a given task. The earliest system was the ILLIAC IV, intended to be a 256-processor system but only one quarter of the machine was ever built. Although great computational power could be achieved on parts of problems, many problems suffered large-scale degradations because of inadequate facilities for moving data amongst the processors.

The attractions of obtaining increased performance by using many computers is obvious, hence the many attempts to develop such systems. To date, the most successful of these have been two designs in the United Kingdom — the DAP from International Computers Ltd and the CLIP IV from University College, London. Six DAPs have been installed since 1980, each with 4,096 processors in a 64×64 array. They are used in a wide range of general-purpose computations. The CLIP is available commercially in various configurations of 32×32 , 32×64 and 64×64 processors.

The processor arrays are specialized in the sense that they all respond to a single instruction stream broadcast from a master processor. Hence, if one processor is performing an addition operation, all the

other processors also perform an addition operation (which might be a dummy). Although this 'lock-step' characteristic appears very restrictive, most user experience has shown that such lock-step processor arrays are highly flexible. Processor arrays and vector processors are often called single instruction multiple datastream (SIMD) processors, as a single instruction produces many results.

It is generally accepted that a collection of processors that did not have to work in lock-step fashion would be more flexible than a SIMD system. Therefore, there are many attempts to develop multiple instruction datastream (MIMD) systems, mainly as part of programmes such as fifth generation ALVEY and ESPRIT. One of the dreams of computer scientists is the ideal MIMD computer with the following properties: it supports a programming methodology that does not need to know how many computers are in the system; it can double in performance if the number

of computers is doubled; and it automatically rejects any failing computer yet continues to work.

The production of computers with these desirable properties is a major research target. Nearly every day there is a new proposal for a computer architecture system which its designers claim will meet the aims. Many one-off experimental systems exist in universities, but none seem to have gained external acceptance. There is, perhaps, an analogy between the programmes to build an ideal MIMD system and that to build an operational fusion power generation plant. Everyone agrees that it would be a good thing to do, lots of money is being thrown at the problem, but nobody has yet shown that it can be done. However, interesting things are happening — watch this space! □

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Quantum fluid dynamics

Instabilities at absolute zero

from K. W. Schwarz

ILLUSTRATIONS of flow around a sphere are a seemingly essential part of every textbook on fluid dynamics. At low velocities, the fluid is seen to make its way smoothly past the sphere. The nonlinear nature of flow phenomena, however, becomes spectacularly evident at higher velocities, with the appearance of a vortex ring (similar to a smoke ring) in the wake of the sphere. At still higher velocities, the ring is intermittently shed into the fluid, leading eventually to completely turbulent flow behind the sphere. McClintock, Bowley and colleagues, on page 797 of this issue¹, examine the quantum mechanical analogue of this venerable problem. In the latest in a series of remarkably elegant experiments, using electrons injected into liquid helium to form bubbles, they find, unexpectedly, that when a bubble is pulled very quickly through the fluid a stream of microscopic quantized vortex rings is generated. The similarity to classical flow around a sphere is both striking and aesthetically satisfying, and it should help us understand better the appearance of instabilities in quantum fluids, such as ⁴He.

Helium-4 remains liquid down to absolute zero. In this limit, it obeys the laws of quantum mechanics, which operate on a macroscopic scale under such conditions. The rules governing the motion of this 'superfluid' are therefore very different from those of classical fluid dynamics, and are still only imperfectly understood. Two kinds of excitations are known to be possible in superfluid ⁴He: vibrational excitations, somewhat analogous to sound waves, and flow excitations in the form of quantized vortex lines. The latter are

essentially localized microscopic whirlpools, the fluid circulating rapidly around a central line about 0.1 nm thick, with the velocity dropping off away from the core. Such quantized vortex lines can exist as arbitrarily configured continuous curves in the fluid, either as segments which terminate on boundaries, or as free-floating loops and rings. Once there, they obey the laws of classical fluid dynamics. What is not understood is how they arise in the first place as an instability of quantum flow. Thus it is of the utmost interest to study how 'superflow' past a simple object such as a sphere becomes unstable.

To carry out their experiments the authors make use of the well-established fact that an excess electron injected into liquid helium forms a well-defined spherical bubble with a radius of about 1.5 nm. Such electron bubbles can be accelerated through the superfluid by applying an electric field, and their motion can be detected as a current. Since the movement of such a structure displaces thousands of helium atoms, the flow around it can be thought of as macroscopic. Yet, the dimensions of the bubbles are so small that any quantum instability that occurs can be expected to involve individual excitations of the kind described above. Earlier investigations^{2,3} had in fact established that, depending on the pressure, a rapidly moving electron bubble will either create a single vortex ring to which it remains attached, or it will emit a continuous stream of vibrational excitations. Thus, depending on circumstances, instability with respect to either type of possible excitation was known to occur.

Although they have also made major contributions to our knowledge of the vibrational-excitation instability⁴, McClintock, Bowley and coworkers have more recently concentrated on the situation where the flow around the bubble becomes unstable leading to the creation of quantized vortex rings, a situation that appears to more closely resemble the classical problem. By doing experiments in isotopically pure ⁴He at sufficiently low temperatures, the scattering of thermal vibrations by the moving bubble becomes rare, so they have been able to study the problem in its purest form. That is, they can look at what happens as the bubble is pulled more and more forcefully through the zero-temperature superfluid. They find that initially the bubble accelerates exactly as would a sphere moving through an ideally frictionless classical fluid⁵. When a critical velocity is reached, a quantized vortex ring attached to the sphere is created. The unexpected result of their latest experiment¹ is that, when a very large field is applied to pull the bubble through the fluid very quickly, a continuous stream of vortex rings is emitted by the moving bubble.

These latest results promise to lead to new insights into the nature of quantized-vortex creation. At present, not much is understood theoretically about this process. An early calculation by Schwarz and Jang⁷ demonstrated that the critical velocity is determined by the instantaneous application of energy and momentum conservation, implying that the vortex appears as the result of a sudden quantum transition. This point of view appears to be generally accepted today and much more ambitious models have recently been proposed^{8,9}. The subject, however, is controversial, and in particular the very basic issue of defining the configuration in which the quantized vortex first appears remains under debate. By comparing experimental results of the kind reported here against hydrodynamic calculations using various starting geometries for the vortices, it may now be possible to determine this initial configuration. Prospects for attaining this milestone seem encouraging, although a great deal more theoretical and experimental effort will be required. □

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Cytoskeleton

Splitting hairs and other intermediate filaments

from Peter M. Steinert and Alasdair C. Steven

EVEN though keratinized tissues rich in intermediate filaments were possibly the first biological substances to be probed by X-ray diffraction techniques (ref. 1), solving their structure remains a plum to be picked. These filaments, 10–15 nm in diameter, form one of the three major cytoskeletal networks in eukaryotic cells. They differ from the other two, F-actin and microtubules, in several important respects. For example, unlike the other two, intermediate filaments are composed of α -helix rich fibrous protein subunits, and their distributions and state of assembly in cells seem to be governed by quite different rules. Their precise function(s) in cells remain obscure, so an understanding of the details of their structure is an important first step in elucidating their cellular functions. A flurry of recent papers describing the amino-acid sequences of around 30 subunits suggests that the broad principles of intermediate filament structure will soon be understood.

All the filaments have been found to contain a central α -helical 'rod' domain of conserved secondary structure, flanked by end domains of variable size and chemical character. The rod domains possess four discrete segments of precisely conserved size that can form coiled-coils, termed 1A, 1B, 2A and 2B, interspersed by regions that cannot form coiled-coils (a in figure). The rod domain sequences conform to several distinct types: the 10–15 acidic keratins (Type I); the 10–15 neutral-basic keratins (Type II); vimentin, desmin and glial fibrillary acidic protein (Type III); and neurofilament proteins (possibly comprising at least one other type³). Convincing evidence now shows that pairs of subunits align in parallel and in axial register to form two-chain coiled-coil molecules (b in figure)² but it is not known how these molecules combine to generate higher orders of intermediate filament structure. Observations using the scanning transmission electron microscope³ suggest that most filaments,

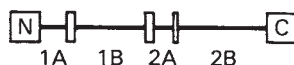
irrespective of their subunit composition, contain about 16 molecules in cross-section. These and other data have also contributed to the emerging concept that the rod domains form the cores of intermediate filaments and that the interactions of these domains are primarily responsible for defining filament structure. The end domains are located on the periphery of this core and define the functions of different filaments in cells^{2,3}.

At the immediately supramolecular level, several lines of evidence invoke a dimeric association of molecules in a four-chain oligomeric complex^{4,5}. It is plausible that such an association should be based

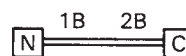
particles, but interpreted them not as models 3–5, but as staggered pairs of four-chain complexes. Again, the complexes might be parallel or antiparallel, but their neighbouring constituent two-chain molecules should nevertheless comply with models shown in c.

The ingenuity of these experiments notwithstanding, their apparent incompatibility poses a problem: which data are correct? Or is every investigator right? Assuming that keratin and Type III intermediate filaments do share a common structure, there may be at least one way to reconcile this paradox. Conceivably, there may be multiple modes of intermolecular associations in fully assembled filaments, so that more than one of the models in c may be applicable. Then, disassembly or reassembly of intermediate filaments under conditions of varying salt or pH might selectively weaken different sets of interactions and split the same structure into different four-chain complexes. This idea could be tested by the

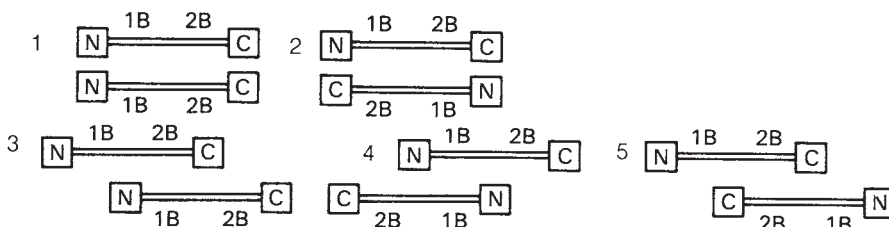
a Subunit model



b Two-chain molecule



c Possible models for four-chain complex



on alignment of the long (1B and 2B) coiled-coil segments⁵; if so, there are essentially only five possibilities (c in figure). Models 1 and 2 predict the segments to be about 48 nm long and the others predict them to be about 70 nm.

Two recent studies have cast some light on these alternatives. Using quick-freeze deep-etching electron microscope techniques, Ip *et al.*⁶ have characterized a 48 nm particle as the tetrameric building block for vimentin. This is consistent with models 1 or 2, but cannot distinguish between them as the differences depend on the polarity of the molecules. But, by using a monoclonal antibody against region 2B of the desmin rod, Geisler *et al.*⁷ seem to have answered this question. They found dumbbell-shaped structures averaging 48 nm in length, denoting antibody binding at both ends of a similar tetrameric desmin complex and compatible only with model 2. However, in other studies on isolated α -helix-enriched particles obtained from proteolysed keratin intermediate filaments, 1B tetramer particles were obtained^{8,9} which could only have derived from models 1 or 4 and not from model 2. Under modified salt conditions, Ip *et al.*⁶ visualized larger 70-nm-long vimentin

experimental approaches described here.

Taken together, recent reports make it likely that neighbouring pairs of two-chain coiled-coil molecules in intermediate filaments are antiparallel, meaning that the filaments are non-polar structures. Thus, unlike the other mainstays of the cytoskeleton, F-actin and microtubules, intermediate filaments don't know whether they are coming or going. Further developments in this rapidly evolving field hopefully will extricate researchers from a similar dilemma. □

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100 Years Ago

MANY interesting excursions have been arranged for the Aberdeen meeting of the British Association. One of them will, of course, be to the great granite quarries in the neighbourhood of Aberdeen. Her Majesty has invited 150 of the members to Balmoral, where they will be shown over the grounds and have lunch. It is not to be expected that the Queen will personally receive all the members, though it is possible that a few representative men of science may be presented to Her Majesty.

From *Nature* **32** 400, 27 August 1885.

Plate tectonics

Room at the top of the world

from J. Tuzo Wilson

BENEATH the ice the Arctic Basin is vast, poorly known and difficult to study. Perhaps the only thing that can be said with certainty is that its geology and geophysics are complex. This lack of information has enabled some scientists to claim that there is no room in the Arctic for the global motions of the Earth's crust required by plate tectonic theory. This raises a dilemma for supporters of that theory, but fortunately a broader view and new data from two expeditions (Weber, J. R. & Sweeney, J. F. *J. geophys. Res.* **90**, 663, 1985; Sweeney, J.R. *Geos* **13**, 8; 1984) suggest alternative solutions.

One aspect of the problem is that most mid-ocean ridges (where new oceanic crust is created) are aligned roughly north-south, spreading out from a ring through the southern oceans (Fig. 1a). This rough symmetry of the mid-ocean ridge system is a relic of the break-up of Gondwana which commenced about 250 Myr ago (Fig. 1b). At that time, the southern continents were clustered round Antarctica. But since the girth of the globe increases towards the Equator, the continents were forced to separate as they moved slowly northwards. This spreading apart of those continents which once surrounded Antarctica like blocks in an arch explains why they all have triangular or trapezoidal shapes narrowing to the south. Once across the Equator, one would expect the continents to be forced together again and to override mid-ocean ridges, just as North America has overridden the East Pacific Rise. If forced to converge in Arctic regions, there would not be enough space

for them or to permit any fresh spreading.

As if not content with the rate of progress towards such a continental crash, dozens of fragments like those of Baja and western California have been moving polewards up the coast of North America. This has created a complex of terranes in Alaska and British Columbia, which led P. B. Jones (*Can. Soc. Petrol. Geol.* **80**, 83; 1982) to suggest that all of Alaska is moving towards the Arctic Basin. But that view is at odds with new data emerging from Canadian expeditions which indicate extension rather than compression in the Arctic. The impasse can be avoided if account is taken of the globe-encircling zone of subduction and continental collision which separates the Gondwana continents from the others. This zone extends from the Mid-Atlantic Ridge near the Azores eastwards along the active mountain and island arcs of southern Eurasia to Indonesia and thence around the rim of the North Pacific Ocean to Central America and the Caribbean (Fig. 1a). The sliver of western California which is now moving north lies on the southern side of this zone. This suggests that rather than pushing into the Arctic Basin, the fragments now forming Alaska could have been accreted at a subduction zone which 'insulated' the Arctic.

An important question in plate tectonics is whether ocean basins are passively pulled apart at mid-ocean ridges, or whether the spreading is driven actively by forces originating in the mantle. D.I. Gough (*Nature* **311**, 428; 1984) has shown that a whole variety of phenomena, ranging from topography to *in situ* stress measurements indicate that active spreading is continuing beneath the western United States in the region which has overridden the East Pacific Rise. This supports the latter possibility, which is lent further credence from recent discoveries in the Arctic Basin.

The chief conclusion from the LOREX (Lomonosov Ridge Expedition) expedition, reported by Weber and Sweeney, is that the Lomonosov Ridge (Fig. 2) is a remarkably uniform, steep-sided, flat-topped feature with at least one kilometre of flat-lying sedimentary layers underlain by a root, extending perhaps to 25 km depth within which the density increases downwards from 2.5 to 2.95 g cm⁻³. This lends support to the widely accepted view that the ridge is a block-faulted fragment of continental margin detached from Siberia 53 Myr ago by normal seafloor spreading at the Nansen-Gakkel Ridge in the Nansen Basin.

Weber and Sweeney suggest from limited data that the Alpha Ridge may once

have been joined to the Lomonosov Ridge and have had a similar origin, but that extension, including faulting, and injection of much mafic rock (as in the Lord Howe Rise north-east of New Zealand) can explain its thinner crust and magnetic lineations. They also admit that it could be a plateau of thick oceanic crust. Other results indicate that the Canadian end of the Makarov Basin is a faulted trough 25–60 km wide produced by extension "other than typical seafloor-spreading". It is partly filled with flat-lying sediments and has a crust 14 km thick.

Preliminary analysis of data gathered on a later expedition — CESAR (Canadian Expedition to Study the Alpha Rise) — reported by Sweeney, strengthens the idea that the Canada Basin also opened by



Fig. 2. Sea floor geography beneath the North Pole, showing the main tectonic features.

spreading when a crustal block, comprising northern Alaska and northwestern Siberia, which had lain against the Canadian Arctic Islands, rotated to its present position. Imprecise age determinations suggest that continental break-up began some time between 131 and 113 Myr ago and that new sea floor was formed from 118 to 79 Myr ago. H. R. Jackson, D. Forsyth, & G. L. Johnson (personal communication) support these views and show that the thickness and seismic velocities beneath the Alpha Ridge correspond to those of the thickened oceanic crust beneath other large oceanic plateaus, including the Manihiki, Shatsky and Hess rises. All these features formed during the magnetically quiet period in the late Cretaceous, probably at complex plate junctions.

The inescapable conclusion to be drawn from these studies is that despite the expectation from global tectonics that the region would be under compression, abundant spreading has in fact occurred. Together with the evidence for active spreading under the western United States, the new data from the North Pole support the view that spreading is caused by powerful forces within the mantle. □

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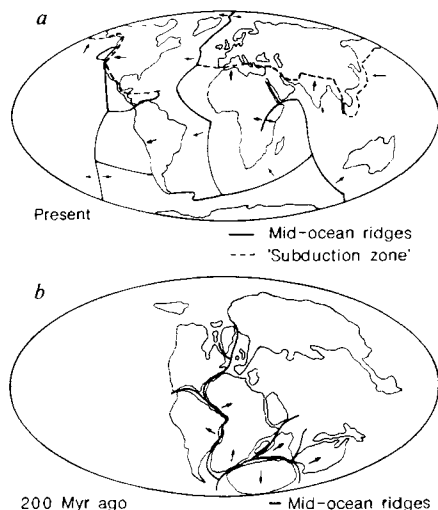


Fig. 1 Cartoon maps of the world as it is now (a) and as it was 200 Myr ago, before the break-up of Gondwana (b). The spreading centre which formed a ring around Antarctica has expanded greatly, driving the continents northwards. Today, a subduction zone decouples the movements of the southern and northern continents.

The fall of Nikolai Vavilov

J.R.S. Fincham

The Vavilov Affair. By Mark Popovsky. *Archon/Clio:1985**. Pp.216. \$22.50, £21.40.

THIS book, by a former Soviet writer now resident in the United States, traces the career, downfall and destruction of the most famous Soviet biologist of the inter-war period. N.I. Vavilov was the son of a wealthy self-made businessman and one of two brothers, the other a physicist, who both became Academicians.

As a young man Vavilov spent two years (1912–1914) in England under the tutelage of the geneticist William Bateson and the plant breeder Rowland Biffen. At the time of the 1917 revolution, when he was 30, he had just been appointed to a professorial chair in Saratov. In 1920 he left Saratov to become head of the Bureau of Applied Biology in Petrograd and five years later was appointed Director of the new All-Union Institute of Applied Biology (in effect, Plant Breeding), the foundation of which, according to Popovsky, was a consequence of Lenin's having read an American book on the great potential of plant breeding. This Institute became the headquarters, and Vavilov the chief, of a great network of research institutes and experimental stations employing, it is said, as many as 20,000 people. With these resources at his disposal, Vavilov devoted his great energies to organizing and leading plant-hunting expeditions to many parts of the world and established very extensive collections of wild relatives of cultivated plants for investigation in his own institutes. He also wrote indefatigably on plant geography, the history of agriculture and origins of cultivated plants, and the theory and practice of plant breeding, with emphasis on the use of wild species as sources of such valuable traits as disease resistance.

From the early 1930s, Vavilov's enterprises came under increasing challenge from the ambitious T.D. Lysenko whose position, which may be summed up as Lamarckist, anti-élitist and ignorant, attracted increasing political support. In the Soviet climate of the time, criticism of Vavilov's science as academic and insufficiently practical slipped easily into accusations of philosophical and ideological error and eventually of treason. Vavilov was arrested while on a collecting expedition in August 1940, and sentenced to death as a saboteur and spy in July 1941, but was not in fact executed; he died in the prison hospital in Saratov in January 1943.

Mark Popovsky started his research into Vavilov's life in 1964. At that time the process of posthumous rehabilitation was already well under way, and Popovsky was able to gain access to a number of different sources. For the period up to 1940 he cites mainly the archives of the Lenin Academy of Agricultural Sciences (from the Presidency of which Vavilov was displaced by Lysenko in 1933) and of Vavilov's old Institute. He also interviewed a considerable number of Vavilov's former colleagues. In 1966 he published a narrative based on this information under the title "A Thousand Days of Academician Vavilov" in a Soviet magazine. This account was cited (with some critical amendment) by Zhores Medvedev in his definitive book *The Rise and Fall of T.D. Lysenko*, published in I.M. Lerner's English translation in 1969.

The most novel part of the present book is based on the massive police files on Vavilov to which Popovsky was rather amazingly granted access. These contained a full record of Vavilov's lengthy interrogation and brief trial, as well as a large collection of much earlier material — letters from Vavilov's opponents and from police informers — and some later letters from Vavilov appealing for clemency. Popovsky describes his daily visits to the procurator general's office in Moscow, where he sat making notes under the eye of an invigilator who allowed him to see more than he was supposed to, assuming, Popovsky conjectures, that a person of such affrontery must be acting on higher

authority. The official purpose of this exercise is not made clear. There was obviously no intention of allowing Popovsky to publicize his findings. Indeed, he subsequently ran into increasingly bad trouble with the authorities for disclosures. In 1977 he was allowed to emigrate, and managed to smuggle some of his notes out with him, much of the rest having already been sent abroad.

So what do we learn from this new evidence? We obtain some insight into the material — a mixture of bigotry and personal malice — from which the charges against Vavilov were fabricated. We also learn, not too surprisingly, that, under pressure of prolonged interrogation, Vavilov was prepared to compromise. He admitted the charges of deliberately leading Soviet plant research along wrong paths and was prepared to incriminate others (already dead) as accomplices in that crime. However, he continued to deny that he had been guilty of espionage. He never lost hope that, even though imprisoned, he would be allowed to resume scientific work, perhaps in the kind of institute of captive scientists described in Solzhenitsyn's *The First Circle*. It appears that in June 1942 Beria had decided to permit this, but that in the confusion of the war the plan was forgotten or countermanded. At all events, seven months later the opportunity was lost and Vavilov was dead.

In the final chapter of his book Popovsky recounts what he was able to find out as a member of a group sent to Saratov by a Commission of Investigation set up by the Soviet Academy of Sciences. This group interviewed, not very fruitfully it appears, some of the prison hospital staff who had charge of Vavilov during his last days. Popovsky devotes several pages to the cause of death, accusing the prison staff of falsely misrepresenting it as pneumonia

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Before the storm — Vavilov in 1928, driving an electric plough on a station belonging to the Central Asian State University. With him is his son Oleg.

when it was really starvation. It hardly seems to matter much, and one supposes that it may well have been a combination of the two; the circumstances must have been conducive to both conditions.

What was Vavilov's standing as a scientist and what did he achieve? In his own time he enjoyed a considerable international reputation, demonstrated by his election as President of the 1939 (Edinburgh) International Congress of Genetics (which he was prevented from attending). This was no doubt in part a gesture of support for a fellow scientist in difficulties, but was also a recognition of his scientific stature, recognition that was reaffirmed in a special session of the 1980 (Moscow) Congress. But, tragically, much of his work was aborted. His plant collections were largely lost and most of the great books that he had planned could never be written. His most important theoretical contributions, the idea of centres of origin of cultivated plants and the Law of Homologous Variation (which may be para-

phrased by saying that related species have similar genes subject to similar kinds of mutation), hardly bulk large in today's textbooks; they have achieved the status of the valid but obvious — the fate of many good ideas in science. Vavilov's commitment to the preservation and exploitation of wild species is now widely shared, and this may well be his most important scientific legacy. But he will probably be most remembered as a victim of the perversion of science in the service of ideology and the struggle for power.

Mark Popovsky has written a most saddening and thought-provoking book. As a work of history it suffers from the defect that its most important sources cannot be checked, either by the reader or now by the author himself. But it is as good a biography of Vavilov as we are likely to get in present circumstances. □

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Something in the air

James E. Lovelock

Chemistry of Atmospheres: An Introduction to the Chemistry of the Atmospheres of Earth, the Planets, and their Satellites. By Richard P. Wayne. *Clarendon: 1985. Pp.361. Hbk £30, \$39.95; pbk £14.95, \$19.95.*

TEXTBOOKS about the atmosphere have until now not fully reflected the new knowledge from space exploration. Just as important, they have failed to observe that the atmosphere is essentially a biological product. These earlier texts stand in science rather as does the Old Testament to a Christian; not wrong, merely unfulfilled. Richard Wayne's admirable book is definitely of the New Testament of atmospheric science.

The book is easy and enjoyable to read. It is a fine piece of craftsmanship, with a most comprehensive bibliography and index, and illustrates with clarity the interactions between the professional practice of physical chemistry and the larger domains of science and the world itself. It is especially refreshing to find illustrations taken where appropriate from sciences ranging from astronomy to zoology. Here the demarcation disputes over the territorial boundaries of science no longer imprison our imagination.

My only point of difference is over the space given and the deference shown towards that curious obsession of aeronomists — impending doom from the depletion of stratospheric ozone. We inhabit a world where the environment is threatened by carbon dioxide increase, changes in land use acid deposition and a rise in the concentration of tropospheric

ozone. To spend more than a few words on the lesser ailment of stratospheric ozone depletion seems out of balance. I well understand why physical chemists are transported by thoughts about the stratosphere and the ozone there. Ozone is a key constituent of an important part of the atmosphere and the stratosphere is an near-ideal reactor for the study of photochemical reactions, no less than 250 of which have so far been listed. It should not be necessary to use doubtful arguments about a possible increase of skin cancer in the next century to justify such important research. This disagreement over emphasis in no way detracted from my enjoyment of the thorough discussion in the book of the intricate chemistry of real or potential pollution problems. There is also proper and decent speculation about consequences and on what more we need to know.

Recent explorations have brought the upper atmosphere down to Earth. The chapters on ionization and on the air glow nicely bring together the old and the new chemistry of this upper region of the air. And the account of extraterrestrial atmospheres describes the very different chemistry of those barren worlds and provides a contrast to that of the Earth where life has created a strange and beautiful anomaly in the Solar System.

Chemistry of Atmospheres is much more than a first-rate student text. It is also a scholarly interim report on the state of the subject as seen by a broad-minded physical chemist. Anyone with a serious interest in the atmosphere and the chemistry of the gaseous particles it carries will benefit greatly from reading Richard Wayne's book. □

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Sweet — and sour

John Yudkin

Sweetness and Power: The Place of Sugar in Modern History. By Sidney W. Mintz. *Viking: 1985. Pp.274. \$20, £14.95.*

DURING the past three centuries, the quantity of sugar consumed in Britain has risen from an average per person of 4 lb a year to the present 100 lb, so that it now constitutes a greater proportion of the diet than does any other food, or indeed a greater proportion than that contributed by all the meat, poultry, eggs and fish that we eat. There have been similar increases in sugar consumption in other industrialized countries, although they began more recently. By itself, sugar would not be consumed in very great quantities, and then only in solution. But in combination its wide range of properties make it suitable for use in sweetmeats and fruit preservation, for enhancing the taste of vegetables and meats, for making jam and ice cream, and for making intricately sculptured designs ranging from the icing on cakes to large sculptures of figures and buildings.

The cultivation of sugar cane began in the Far East about 500 BC and during the seventeenth and eighteenth centuries in particular spread rapidly to many parts of the world where the climate is tropical or sub-tropical. Because it needs intensive labour, the growing of cane as a crop resulted in a massive movement of populations and was responsible for the resettling of great numbers of Africans in the Caribbean and central and north America, and for the extinction of the indigenous population of several of the Caribbean islands. It is difficult to exaggerate the demographic, cultural, social and economic effects of the importation of black slaves to the Western hemisphere. Among other things, it was responsible for the dependence of the sugar-growing countries on what virtually became a monoculture, and thus for the distortion of local agriculture, local food supplies and local patterns of nutritional deficiencies.

What was the cause of the enormous increase of sugar growing in these countries and its consumption both there and in the industrial nations, beginning with Britain? In his wide-ranging account of the history of the subject, Dr Mintz mentions such factors as rapid developments in the efficiency of production of the cane and its yield of sugar, technical improvements in refining, the introduction of sugar from beet, the spread of the drinking of hot beverages — tea, coffee and chocolate — as well as the discovery of eating chocolate, and, finally, the rise in the purchasing power of individuals in the consumer nations. He also convincingly develops the theory that production and

distribution of sugar powerfully affected the relationship between the wealthy and the labouring classes: "Probably no single food commodity on the world market has been subjected to so much politicking as sugar". This is a view that, in more lurid detail, is exposed by Al Imfeld in his recent book *Zucker* (unfortunately not yet translated from the German). Together with *Sweetness and Power*, these accounts serve as an illustration of the extent to which the study of this universally popular food is the province of the historian, economist, demographer, geographer, anthropologist and sociologist, as well as the clinician and scientist.

As a final attractive titbit, Dr Mintz



Enough to make you sick? Etienne Tholoni, a great French sugar baker, puts the finishing touches to a life-size chocolate nude with spun-sugar hair. She is lying on a bed of six-hundred sugar roses. (Centre de Documentation du Sucre; reproduced from *Sweetness and Power*.)

serves up a splendid dessert in which he discusses the current changes in the traditional eating patterns of industrialized societies, and the extent to which the multifarious uses of sugar play a part. The wide and increased proportion of specialized manufactured food and snacks, and the fast-food revolution, have changed when, where, what, how much and how quickly people eat, and the result is to produce what Dr Mintz calls "desocialized eating".

This is a book that can be read for its fascinating story or studied as a well-researched, scholarly and excellently documented work; it is beautifully written, with a good index, copious bibliography and extensive notes. Such a combination of qualities is a rare commodity — unlike the ubiquitous substance that is the subject of the book. □

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Eating for survival in China

Brian Bertram

The Giant Pandas of Wolong. By George B. Schaller, Hu Jinchu, Pan Wenshi and Zhu Jing. *University of Chicago Press*: 1985. Pp.298. \$25, £23.75.

WE HAVE been waiting for years for data on giant pandas in the wild in China. A full-scale collaborative research programme in the field started there at the end of 1980; it is still continuing, but the results of the first 18 months of work have now been published.

George Schaller is renowned for his ability to obtain superb data from large wild mammals — gorillas, lions, snow leopards — under difficult conditions. With his work on giant pandas the tradition continues. As the report makes clear without emphasizing the point, pandas are exceedingly difficult creatures to study. Quite apart from the administrative, logistical and language problems involved, the animals inhabit dense bamboo forest on steep mountainsides. The climate is unlovely: "... cool and wet ... snow fell between late October and April ... on rare days when the rainclouds part and sun reaches the valley ... even when it did not rain, fog continued to swirl along slopes, everything remained sodden ...". And the quiet, shy and relatively inactive pandas cannot be seen through the bamboo if more than 10–15 metres away, and they do their best to remain further away than that. During a 16-month preparatory phase, the research team saw pandas only 39 times. Only radio-tracking makes them at all possible to study, but still not at all easy.

The team had radio-collars on six individuals, and so managed to get information from over 1,500 panda locations. In the three main data chapters they present the results. The hard-earned information is fascinating, but pandas basically are not exciting for the very good reason that their lives are largely taken up with eating bamboo (over 99 per cent of their diet). Nonetheless, the book brings out beautifully the complex and highly unusual interaction between the panda and its food supply.

Giant pandas, as carnivores in origin, have a short gut in relation to their body size and to their food needs, and they cannot break down cellulose. Digestion is inefficient, and the food passes through the gut rapidly. A 90 kg panda has to take in between 12 and 38 kg of bamboo per day; the amounts vary with the season, and depend on whether the panda is feeding on leaves (in summer), leaves and young stems (in winter) or old stems and new shoots (in spring). It defaecates about 48 times per day, the faeces totalling 20 kg

or more in weight. The nutrient level of bamboo apparently remains relatively constant through the year, but while bamboo is always abundant it is time-consuming to gather. Protein intake levels seem adequate, and the prodigious amounts consumed are probably required to obtain enough digestible carbohydrate, particularly in spring, the lean season for pandas. At this time of the year, the limitation on the animals' intake of food may be their guts' ability to deal with the bulk.

A panda in the Wolong study area spends most of its life taking in food. It forages for 13 hours a day, rests (conserving scarce energy) for 10 hours, and fits everything else (which is not much — drinking, travelling, grooming, defaecating, scent-marking, playing and socializing) into the one remaining hour. It is no wonder therefore that the home ranges and the daily distances travelled are both small, and the animals very largely solitary.

One chapter brings together the information on reproduction, breeding behaviour and communication from captive studies in zoos and from this first major field study. There is probably delayed implantation; this is suggested by the extraordinarily variable gestation period (ranging between 97 and 163 days in fewer than 30 measured pregnancies in captivity), the time lag after copulation before a progesterone rise, and the minute size (90–130 g) of the one or two offspring at birth. At about 0.011 per cent of maternal weight, these are the smallest eutherian mammal babies known.

Anatomical, physiological and behavioural features are used to re-examine the old problem of giant panda phylogeny. The species appears in many independent respects to be quite closely related to the bears, whereas the much smaller red panda seems to be close to the Procyonids (the raccoon and its relatives). But in many other respects giant pandas seem to be merely enlarged red pandas and phylogenetically close to them. There has clearly been a much greater degree of convergence in evolution than is comfortable to taxonomists. The authors' solution to the contradictions is the more usual one of considering red and giant pandas to be the closest relatives, and therefore of necessity distancing both from both bears and raccoons. This is not the conclusion of the most recent molecular studies in which O'Brien *et al.* (*Nature*, in press) have asserted that the giant panda is close to the bears and the red panda close to nothing else.

The data on giant pandas in the wild will be useful in managing the small wild population, estimated at about 1,100, mostly in a few circumscribed reserves. But equally valuable will be the fact that the study has focused international interest on the plight of the giant panda and on the steps which will need to be taken

for the conservation of the species and its habitat.

The long-cycle flowering and subsequent temporary die-off of bamboo, and consequent starvation of pandas, has received a great deal of publicity. The situation is very complex. In "proper" panda habitat, with a variety of bamboo species, each flowering intermittently but asynchronously, the pandas' food supply does not disappear. But human pressure has in particular resulted in the loss of the lower-altitude species of bamboo in many areas, in effect removing the pandas' emergency alternative rations. The animals can and do switch between different bamboo food types, as this study showed; but it also showed that they have very little spare time to spend in searching for and gathering their food. They are near the limits of their existence, for several reasons. The remedies for the situation will form the basis of the research team's next report. We shall await it just as eagerly. □

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Matter of a letter

Stillman Drake

The Galileo Affair: A Meeting of Faith and Science. Edited by G. V. Coyne, M. Heller and J. Życiński. *Specola Vaticana, V-00120 Stato Città Del Vaticano, Vatican City State: 1985. Pp.179. Pbk \$7 (individuals), \$9 (libraries).*

IN 1979 the Pope expressed concern that Galileo had suffered at the hands of persons and instrumentalities of the Catholic Church. Citing a passage from Galileo's open letter of 1615 on religion and science, addressed to the Grand Duchess of Tuscany, he implied that Galileo's theological position was sounder than that of his judges at his trial for suspected heresy, in 1633. The Pope proposed a thorough re-examination of the Galileo affair, placing blame wherever it historically belonged.

Several publications at Vatican City have subsequently appeared. Among the first was the complete official text of all documents relating to the affair now extant at Rome. Most recent is this book, the proceedings of a conference held at Cracow in 1984 and attended by historians, philosophers, scientists and theologians. History of the Galileo affair occupies the first section, followed by sections on Galileo and science, and on cultural repercussions of the Galileo affair.

As translator of Galileo's letter of 1615 and of his books, I oppose the claim (p.30) that the former "is filled with assertions that the Earth's motion, both diurnal and annual, can be conclusively proved by

necessary demonstrations based on sense experiences". No such categorical assertion was made. The part of the letter cited says no more than that after decades of unopposed study, Copernican astronomy was being attacked by philosophers just when it was beginning to appear well-founded by manifest experiences (telescopic) and necessary demonstrations (mathematical).

One such set of foundations was the phases of Venus announced at the beginning of 1611. Another was eclipses of Jupiter's satellites, published in 1613 with some predictions by Galileo and his remark that motion of the Earth had to be considered in calculating them. His calculations showed that the Earth was not at, or even near, the centre of Jupiter's orbit. The scholar concerned is also mistaken in assuming that Galileo alluded to his explanation of tides, which involved no measurements, mathematical calculations or predictions subsequently verified observationally.

In 1615 Galileo openly stated his Copernican belief; no action had yet been taken by theologians on the matter. The purpose of his letter was to show that any action would be untimely, not to prove any scientific conclusion. In 1616 an edict regulating Copernican books was issued. Thereafter Galileo discussed the matter only hypothetically, having promised in his letter to abide by Church action if, contrary to his advice, any were taken.

Thorough re-examination of the Galileo affair as proposed by the Pope is

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still far from accomplished. Another scholar at Cracow writes (p.137): "The revolutionary ideas of Galilean astronomy were due to fortunate conjectures and insights which had no critical empirical confirmation before 1838". No scientist altered his astronomy because of Bessel's observations. Moreover Galileo's calculations of satellite eclipses were not fortunate conjectures. Whether philosophers should reject them as critical empirical confirmation of the Earth's place and motions is at least questionable. Philosophy and rhetoric are no longer science, as they were until Galileo separated them from it.

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AIDS: the scientific story so far

June Goodfield

A Strange Virus of Unknown Origin. By Jacques Leibowitch. Translated by Richard C. Howard. *Available Press (Ballantine, New York): 1985. Pp.172. Pbk \$4.95 (United States), \$6.50 (Canada).*

ACQUIRED immune deficiency syndrome, AIDS, struck medical consciousness at a time when, as Robert C. Gallo points out in his foreword to this book, prominent scientists were predicting the end of the great human infectious epidemics. Though the disease had certainly already been present in a few special localities, it appeared where it might have been least expected, namely in technologically advanced, relatively healthy societies.

The medical community might have been taken by surprise, but the cause of AIDS was discovered with a speed probably unparalleled in the history of infectious diseases. A new virus, unique to human beings, preferentially attacks the helper T-lymphocytes, the very cells whose activity is crucial in the mounting of an adequate immune response. The immune deficiency thus provoked lays the victim open to a whole variety of invariably fatal opportunistic infections. As they watch the spread of AIDS gather momentum, medical scientists are realizing that, far from being over, a new, dangerous period of infectious epidemics may just be starting.

We are fortunate that a scientist involved in research into this disease from its very beginning has now written this excellent, slim book. Jacques Leibowitch is a clinical immunologist who has been both close to the problem and totally committed to its solution. Here he successfully demystifies AIDS, recognizing, as we all should, that to know your enemy well is the first step towards victory. Taking us on a survey through the history and origins of the epidemic, he presents a comprehensive and authoritative clinical and epidemiological picture that culminates with sections on prevention and the prospects for treatment — not at all bright as of now.

Everyone from professional scientists to the general public will find this book of great value. It is marred, however, by one stylistic fault. Though at the beginning the writing is fluent and witty, the book finishes in a series of choppy, condensed paragraphs covered liberally by sub-headings, so that it takes on the quality of a briefing paper for a select committee. □

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Rural poverty breeds fertility

from Alok Vajpayee

The conditions of village life in rural India create the need for large families. Reduced fertility will come only with improvement.

THE Family Planning Programme (now known as the Family Welfare Programme) has been in operation in India since 1953. The main approach is to fix targets for sterilization operations and other methods of contraception for each area and to press the responsible authorities to achieve them.

This approach is unnatural, because most villagers in India need large families. Motivating them to adopt family planning is a difficult task. The more primitive the way of life the larger the family they need. This is a matter of survival, not simply an option as many villagers well realize.

Of course, the villages are changing, but very slowly.

The urbanization of the villages is followed by a change in life style. And when a village becomes a town the residents will need smaller families. The rural population of India resides in some 576,000 villages, 55 per cent of which contain fewer than 500 persons and 23 per cent of which have between 500 and 1,000 persons (these data and others in this article are from *Health Statistics of India — 1984*, published by the Ministry of Health and Family Welfare). The smaller the village, the greater will be the need for a large family.

Rural life is harder than urban life, which is why a rural family needs more working hands. In the villages, a family must itself do almost all the work required for its survival. In other words, a rural family is more self-sufficient than an urban family. Take, for example, water supply and the disposal of domestic liquid and solid wastes. For both tasks, an urban family does not have to exert itself at all. Water is supplied to houses through pipes and, for the disposal of liquid wastes and excreta, there is a public sewer system.

Services

In villages, these tasks demand careful attention. According to official statistics, in 1984 some 83,000 villages were without even a single source of year-round drinking water. People have to go and carry water, sometimes from a source miles away from the home. For defaecation, almost 100 per cent of the rural population goes to the fields, outside the villages. In the absence of a proper drainage system and a piped-water supply, because of the space available in village houses, the financial constraints and the lack of technical expertise in the villages for the con-

struction and maintenance of sanitary latrines, it is better for them to go into the open for defaecation than to construct and maintain a latrine in their houses.

In the cities one can purchase all types of services; there are laundry services for washing clothes, crèches to look after the children . . . and many other types of service. In the villages, by contrast, such services cannot be purchased; instead, those considered essential must be performed by the family itself.

Electricity makes life extremely comfortable and saves much human and animal labour. But by 1983, about 275,000 villages had not been electrified while even in those with electricity, the supply is erratic and is frequently interrupted.

Agriculture is the main occupation of the villagers and needs a great deal of human labour, at least as practised by most Indian farmers. For the purchase of household items, clothes, utensils and so on — the majority of villagers depend on weekly markets which are held in larger villages, or they may go to a nearby city. But there is a great problem of transport. Of the villages of India, 53 per cent were not connected by all-weather roads in 1982.

So therefore, all of the tasks performed by a rural family require a great deal of human labour and time, and only a large family is suitable for such a life style.

In the villages, life and property are much more insecure than in the cities. Robberies, land disputes, murders, thefts and familial rivalries are common, but the nearest police station is usually too far away to be contacted quickly, while the law enforcement agencies are lax. At any one time, most of the police force is engaged on law enforcement in the cities, handling strikes and regulating traffic, for example. Even the mass media have an urban bias. A quarrel or theft in a metropolitan city may hit the headlines of a national daily while a major atrocity on villagers may go unnoticed. Most of the national newspapers and magazines suffer from myopia.

A large family gives a sense of security against such happenings as well as 'insurance' against natural calamities such as floods, fires and the like.

The very old and the very young, the sick and pregnant need special care and attention. A large family can deal with sudden crises such as illness and the normal stresses of birth and old age without

help from outside. A young grandchild may be assigned the duty of watching grandma and helping her, or vice versa.

In the villages, professional expert help cannot be easily obtained. According to an estimate made in 1979–80, only 10–15 per cent of births in India were conducted by trained attendants. Most of the rural population depends on quacks for treatment.

Mortality

Inevitably, the death rate is higher in the villages than the cities. In 1982, the crude death rate for the rural population was 12.9 per 1,000 population, while in the urban areas it was 7.4 per 1,000 population. There is a positive correlation between death rate and birth rate; the higher the mortality, the greater will be the fertility. The collapse or removal of a family member in a small family of four or five, with only a few relatives, will have far more serious consequences than in a large 'unplanned' family.

Mortality in childhood, especially infant mortality and under-five mortality, is very high in the villages. The infant mortality rate in 1980 was 124 per 1,000 live births in the villages and 65 per 1,000 live births in urban areas. The under-five mortality rate in 1980 was 46.1 per 1,000 for rural areas and 22.2 for the urban areas. The smaller the chances of survival of a child, the higher will be fertility. Villagers feel that they should produce too many children if they are to be sure that at least one or two sons will survive to provide care for their parents in old age.

So if the government wishes family planning to be a success rather than a mere slogan, it must provide intensive maternal and child health facilities, giving sufficient health coverage to the mother and her new-born. Child-health is the best motivator of family planning.

As things are, the villagers of India need large families. Overall socio-economic uplift and rural development will bring about a change in life-style that will diminish the need and will ultimately reduce fertility. Rural electrification, better transport facilities for rural areas, the construction of good roads, better medical facilities, the provision of a piped water supply and the like are thus the long-term agents of demographic change. □

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Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins

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The enzymatic machinery that carries out RNA synthesis provides the cell with the means to adjust the patterns of transcription in response to environmental and developmental signals. In eukaryotes, this regulation is mediated in part by promoter-specific transcription factors, which are DNA-binding proteins with the ability to discriminate between distinctive DNA sequence elements found in the promoter regions of different genes. The presence of these factors bound to DNA enables other components of the transcriptional machinery, including the RNA polymerase, to initiate transcription with selectivity and accuracy.

THE principal method of identifying the DNA sequence elements that control transcription in eukaryotes has been to mutagenize DNA sequences near the start of transcription, then to test the altered templates either by reintroduction into the cell or by introduction into some experimental substitute, such as an *in vitro* transcription system. Accumulation of new RNA or protein is measured after a period of time, and from this an inference is made about the effect of sequence changes on promoter strength. These studies have shown that, although promoters for protein-coding genes vary in sequence, they are organized according to a common plan (Fig. 1).

Many promoters have an element called a TATA box, with a consensus sequence 'TATAAA', located 25–30 base pairs (bp) upstream from the beginning of the transcribed sequence (for review, see ref. 1). Mutation in this region generates 5' (upstream) heterogeneity in the transcripts^{2–4}, although the overall level of RNA synthesis may not be significantly reduced. Further upstream from the start site of transcription is a region containing one or more additional promoter elements. Some upstream elements, such as the 'CCAAT' or 'GGGCGG' homologies, have now been found in many different promoters, whereas others, such as the metallothionein metal regulatory element^{5–7}, or the heat-shock regulatory element⁸, have a more specialized role. As we shall establish, upstream promoter elements are a principal target for the action of promoter-specific transcription factors.

The activity of many promoters is modulated by an enhancer, which is a separate regulatory element. The enhancer must be on the same molecule of DNA, but can be 1,000 bp or more from the promoter, and may be located either upstream or downstream from the transcription start site (Fig. 1)^{9–12}. The prototype enhancer is found in simian virus 40 (SV40), a DNA tumour virus^{9,10,12–14}, but control elements with similar properties have since been found in other viruses and in the cellular genome (for example, refs 11, 15–17). Some enhancers are tissue-specific^{18–26}, whereas others mediate transcriptional responses of certain genes to steroid hormones²⁷.

Protein factors in transcription

The understanding of promoter function gained by analysis of DNA sequences has certain limitations, which can be overcome only through identification, purification and characterization of the proteins required for transcription. One would like to know how the proteins that bind to promoter and enhancer elements bring about transcriptional regulation and, in particular, to understand how the abundance and activity of transcription factors change through cell growth and development.

The enzyme responsible for mRNA synthesis, RNA polymerase II, differs from prokaryotic RNA polymerase holoenzyme in that it seems to lack any inherent ability to recognize promoters in an *in vitro* reaction. It has recently become clear that crude extracts from eukaryotic cells contain factors that impart promoter specificity to the purified RNA polymerase II and allow *in vitro* initiation of transcription at the same start sites as are used *in vivo*^{28–33}. Some of these factors seem to be required for initiation at all promoters^{34–40}, whereas others are required for initiation at certain promoters but not others. Many of these latter, promoter-specific, factors bind DNA sequences in their respective target promoters. DNase footprinting⁴¹ has been used to measure this site-specific binding in several instances^{39,40,42}. In the ideal case, the binding and transcriptional properties of a factor can be related experimentally, with stimulation of transcription requiring sequences within the binding site, and moreover, with binding and transcription stimulatory activities co-purifying during fractionation of the extract.

The promoter-specific factor Sp1

Sp1, a protein possessing many of the characteristics described above, is a factor obtained from cultured human cells and required for transcription of SV40 (ref. 37). Activation of transcription by Sp1 requires a well-defined upstream element, which in SV40 occupies a region 50–100 bp upstream from the start site for early viral RNA transcription^{14,43–45}. When this sequence is deleted, the early promoter is no longer able to respond to Sp1 (ref. 42) and, inversely, when the sequence is fused to a heterologous TATA box element, the hybrid promoter becomes Sp1-responsive (D. Gidoni and R.T., unpublished). The Sp1 target element seems to function bidirectionally⁴⁶, and plays a role in directing late viral transcription from the opposite strand of the DNA^{14,42,45,47–51}.

Binding experiments show that purified Sp1 interacts directly with the sequences required for the transcriptional response⁴². The sequence asymmetry of the Sp1-binding site is a distinctive feature; unlike the binding sites for prokaryotic transcription

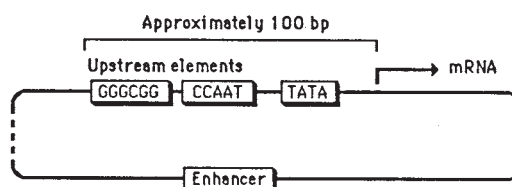


Fig. 1 Arrangement of DNA sequence elements controlling transcription of a typical eukaryotic protein-coding gene. The enhancer may be located upstream or downstream of the site of transcriptional initiation.

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factors, such as the lactose operon repressor, the phage λ repressor, or cyclic AMP receptor protein (for review, see ref. 52), there is no suggestion of dyad symmetry in the region. Sp1-DNA contacts detected by dimethylsulphate protection fall within a sixfold repeated sequence moiety ('GGGCGG'; ref. 53). Sp1 forms strong contacts with three of these GC boxes and weakly contacts two others (Fig. 2). Contacts have so far been detected only in the major groove of the DNA and on one strand. Alteration of individual GC boxes by oligonucleotide-directed mutagenesis suggests the presence of five independently bound protomers within the overall region, with each Sp1 protomer contacting one GC box and protecting ~20 bp from DNase (D. Gidoni, J. Kadonaga, H. Barrera-Saldaña, K. Takahashi, P. Chambon and R.T., unpublished).

Other Sp1 binding sites

When Sp1 was first isolated, its interaction with SV40 was thought to be unique. Recently, however, Sp1 has been shown to be required for *in vitro* transcription of several genes of herpes simplex I virus, including immediate early gene 3 (ref. 95) and a delayed early gene, thymidine kinase⁹⁶. The pattern of Sp1 binding differs from that in SV40, with each control region in the herpes virus containing multiple separate 'unit sites' for Sp1 binding (Fig. 3). Each unit site covers ~20 bp and presumably accommodates a single Sp1 protomer. Binding at the thymidine kinase promoter is particularly interesting because Sp1 seems to interact with the 'distal elements' previously defined by mutational analysis^{54,96}. Activation seems to require both Sp1 and a second, as yet unidentified, factor that binds in the region between two Sp1-binding sites.

In addition to these viral genes, several cellular sequences also interact with Sp1. A DNA segment from the monkey genome, which functions *in vivo* as a bidirectional promoter element⁵⁵, contains two Sp1-binding regions, one of which, the β -site, is shown in Fig. 3⁵⁶. Multiple Sp1-binding sites are also found in 5' flanking sequences of the mouse dihydrofolate reductase (*dhfr*) gene (W.S.D., S. Sazer, R.T. and R. Schimke, unpublished; for sequence, see ref. 57). The monkey β -binding region is relatively large and may be a complex binding site, like that of SV40. The mouse *dhfr* promoter, by contrast, shows unit binding sites reminiscent of those found in the herpesvirus promoters. All the Sp1-binding regions contain one or more perfect copies of the GC box hexanucleotide GGGCGG, which may be present in either orientation with respect to transcription. Not all GC boxes bind Sp1 equally well, and sequences outside the core hexanucleotide seem to modulate the efficiency of binding. A consensus sequence, compiled from 19 individual strong binding sites, is shown in Fig. 3⁹⁷.

Recently a number of additional GC box-containing promoters have been described. These include promoters for cellular genes, such as human metallothionein-I_A (ref. 58), hamster hydroxymethyl-glutaryl coenzyme A reductase⁵⁹, mouse hypoxanthine phosphoribosyl transferase⁶⁰, mouse adenine phosphoribosyl transferase⁶¹, human adenosine deaminase⁶², and rat type II procollagen⁶³, as well as promoters for other viral genes, including herpes simplex I immediate early genes 4 and 5 (ref. 95) and AIDS retrovirus (refs 64-66; K. Jones, J. Kadonaga, P. Luciw and R.T., unpublished). Some of these putative binding sites have been tested in our laboratory for their ability to bind Sp1, but in most cases binding is merely predicted from the DNA sequence.

No shared regulatory feature is evident among the diverse promoters in which actual or potential Sp1-binding sites have been found, and thus we cannot at present explain why some promoters have Sp1-binding sites and others do not. It is possible that Sp1 provides a framework to which other regulatory proteins may bind; it is also possible that Sp1 provides a basal, constitutive level of transcription that is then modulated by other positive or negative regulatory factors. The occurrence of binding sites in the transcription control regions of unrelated viruses suggests that Sp1 is not only widespread among different cells but also

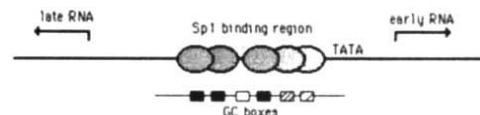


Fig. 2 The Sp1-binding region of SV40. Ellipses represent Sp1 molecules; darker shading indicates higher-affinity binding. Filled GC box moieties are fully protected from dimethylsulphate modification, the hatched GC boxes are partially protected and open GC boxes are unprotected. The initiation sites for late RNA are heterogeneous; only the most Sp1-proximal is drawn.

readily available within the cell to facilitate rapid viral gene expression on infection.

Heat-shock transcription factor

A factor that is similar enzymatically to Sp1 is involved in directing the heat-shock transcriptional response. When cells of any organism are exposed to abnormally high temperature, several heat-shock mRNAs are induced. In *Drosophila*, and apparently in mammalian cells as well, the induction of transcription is mediated by a conserved heat-shock regulatory sequence found in the upstream region of the promoters for heat-shock RNAs (ref. 8; for review, see ref. 67). A heat-shock transcription factor (HSTF) has been isolated from *Drosophila* that seems to interact with this element in a manner analogous to the interaction between Sp1 and its recognition sequence⁴⁰. In the promoter for the relative molecular mass (M_r) 70,000 heat-shock protein, HSTF binds 40-95 bp upstream from the TATA box (Fig. 4). Like Sp1, the HSTF binds to its target sequence in the absence of RNA polymerase II and is promoter-specific, stimulating transcription of the heat-shock gene but not, for example, the actin 5C gene⁴⁰.

One of the attractive features of the heat-shock system is that the *in vivo* role of the factor-sequence interaction has also been analysed. Digestion of chromatin with exonuclease indicates that sequences in the HSTF binding site are accessible in normal cells, but protected, presumably by HSTF, in heat-shocked material^{68,69}. HSTF can be isolated in more-or-less equivalent amounts from normal and heat-shocked cells; therefore, its appearance at the promoter during the heat-shock response probably reflects a change in localization or properties rather than *de novo* synthesis. The nature and mechanism of this change remains to be elucidated.

Other DNA binding factors

The degree to which the effects of upstream elements are reproduced *in vitro* varies depending on the promoter and the way in which the extract is made⁷⁰. With some promoters, such as rabbit β -globin, upstream sequences required for *in vivo* expression have had no effect *in vitro*⁷¹. In other instances, upstream sequences have had a substantial effect on *in vitro* transcription. In addition to the promoters mentioned specifically above, upstream sequences have been shown to be required for maximal *in vitro* transcription of the adenovirus-2 major late promoter^{70,72}, the silkworm fibroin promoter⁷³, one of the sea urchin histone promoters⁷⁴ and the *Drosophila* alcohol dehydrogenase promoter⁷⁵. The dependence on upstream sequences implies the presence of corresponding factors in cell extracts, and at least in the case of the alcohol dehydrogenase and adenovirus promoters, it has been possible to detect the binding of these factors directly, by DNase footprinting (ref. 75, W. Morgan and R.T., unpublished; R. Roeder, personal communication). Although not yet correlated with an upstream element, the elevated histone promoter transcription seen with extracts from S-phase cells may reflect the functioning of yet another promoter-specific factor⁷⁶.

In addition to proteins that bind upstream sequences, a factor that binds the region containing the TATA element has been

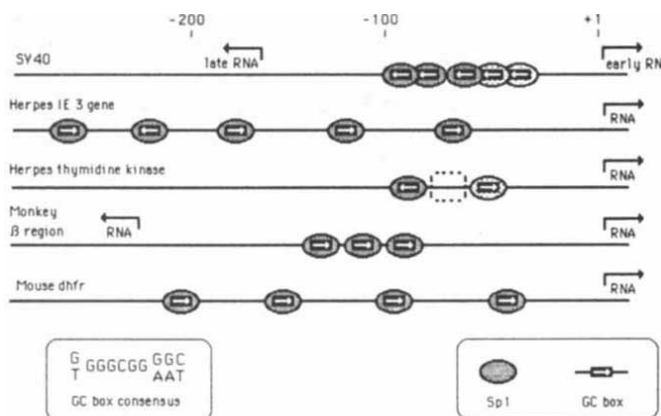


Fig. 3 Different arrangements of Sp1-binding sites. Ellipses represent Sp1 molecules, with darker shading indicating higher affinity binding. Arrows within Sp1 symbols represent GC boxes, with the arrow pointing in the 5' → 3' direction on the G-rich strand of DNA. For clarity, only those GC boxes that actually bind Sp1 are drawn. Some promoter regions have additional GC boxes that do not bind Sp1, possibly because of steric hindrance from Sp1 at adjacent sites. The GC box consensus sequence was obtained by comparison of 19 strong binding sites⁹⁷. Dotted rectangle in herpes thymidine kinase gene represents an additional factor binding between Sp1 molecules.

identified both in *Drosophila*³⁹ and in mammalian cell extracts (ref. 77; W. Morgan and R.T., unpublished; M. Sawodogo and R. Roeder, personal communication). The *Drosophila* factor interacts with several *Drosophila* promoters, including those of the actin, histone H3 and histone H4 genes³⁹. Binding (as assayed by DNase footprinting) occurs in the absence of upstream factors, with the protected region extending from about -40 to +25.

Initiation of transcription

In addition to the site-specific DNA-binding proteins described here the actual initiation of transcription requires RNA polymerase II and at least three other enzymatic factors (refs 34–39; D. Reinberg and R. Roeder, personal communication). Under appropriate conditions, these proteins form a complex at the promoter before initiation of transcription.

Various kinetic and order-of-addition experiments have been of some help in elucidating the steps involved in complex assembly. Davison and coworkers⁷⁷ showed by an indirect depletion assay in a mammalian system that DNA fragments containing a functional TATA box homology could sequester an essential transcription factor from the reaction. Fire and coworkers⁷⁸ carried this approach further and developed a model for ordered assembly of a complex at the adenovirus major late promoter. Their results suggest that factors present in two fractions bind first, in an indistinguishable order, followed by association of RNA polymerase II and, in a final rapid step, factors present in a third fraction. In the absence of nucleoside triphosphates, no initiation occurs and the complexes are stable for at least an hour. Although it is important to interpret these experiments cautiously, because separation and purification of the components might cause events to happen differently *in vitro* than in the cell, this sort of experiment seems to be the best available tool for elucidating the roles of the multiple factors that participate in the initiation reaction.

A marked inhibition of RNA polymerase II activity is seen when β - γ -imido ATP (AMP-PNP) is substituted for ATP in the promoter-directed *in vitro* transcription reaction⁷⁹. The ATP analogue, which cannot be hydrolysed at the β - γ position, apparently fails to satisfy a requirement for hydrolysable adenosine nucleotide during the initiation reaction. AMP-PNP can be hydrolysed normally at the α - β position, and in the post-initiation elongation phase of the reaction can be incorporated normally into RNA. Addition of deoxy ATP to a reaction containing AMP-PNP restores the ability to initiate even though

the deoxy compound cannot be incorporated into the nascent RNA chain^{37,80}, clearly demonstrating that the requirement for β - γ hydrolysis is separate from elongation. The requirement for a hydrolysable adenosine compound is independent of which base is present at the 5' terminal position in the RNA, and has been seen only with RNA polymerase II and not with the other eukaryotic RNA polymerases. As yet, it is not known whether hydrolysis is carried out by the polymerase itself, or by one of the additional factors involved in initiation.

How factors influence transcription

The role of upstream factors should be viewed in the context of their interaction with other components of the transcriptional machinery. In the simplest case, an upstream factor might bind next to the transcriptional initiation site, thus inducing a conformational change in either the DNA or in other proteins that make up the initiation complex (Fig. 5a). An example of a promoter that may be activated by this mechanism is that for the *Drosophila* M_r 70,000 heat-shock protein, where the most proximal heat-shock recognition sequence is only 15 bp from the TATA box. In other heat-shock promoters, however, as well as many of the Sp1 responsive promoters (refs 67, 81; see also Fig. 3) the upstream factor binding site may be situated farther from the initiation site, making direct protein-protein contact less likely as a mechanism for transcriptional activation. Moreover, many promoters have multiple binding sites for upstream factors, with the most distal sites clearly out of range for direct contact with proteins bound at the initiation site.

There are several possible models that could explain the apparent ability of upstream factors to act at a distance. After initial binding to the recognition site, a factor might shift positions to make productive contact with other proteins at nearby sites (see Fig. 5b). A necessary consequence of this model is that an input of energy would probably be required to allow the factor to dissociate from its high-affinity binding site and move along the helix. An alternative model is that the upstream factor might remain in place but induce a conformational change in the DNA helix, such as an unwinding; in this case, the arrow in Fig. 5b can be taken to represent the propagation of the conformational strain through the DNA. A different possibility is drawn in Fig. 5c, where a second factor binds alongside the first to create a continuous scaffold of protein. This interstitial binding may occur in herpes thymidine kinase, where a second factor has recently been found to bind between the two Sp1 sites (see Fig. 3). Another model supposes that factors bound at multiple upstream sites allow DNA to condense into a compact nucleoprotein structure (Fig. 5d). There is as yet no direct evidence for such a structure, although similar models have been proposed to explain the role of multiple binding sites involved in site-specific recombination and DNA replication in bacteria⁸² and for enhancer activation of promoters in eukaryotes⁸³.

Some upstream elements function bidirectionally. This might be expected with the heat-shock element, where the consensus recognition sequence has a dyad symmetry. Similarly, in herpes thymidine kinase, two Sp1 binding sites are present in opposite orientations, making it easy to explain the activity of the upstream elements when their overall orientation is inverted⁸⁴. The properties of the SV40/Sp1-binding region are more difficult

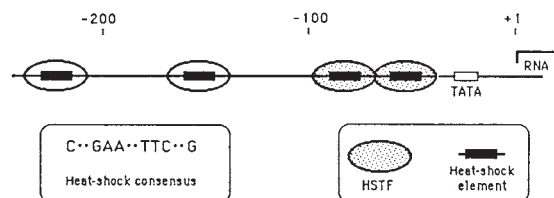


Fig. 4 Arrangement of HSTF binding sites in the promoter for the *Drosophila* M_r 70,000 heat-shock protein. Stippled ellipses cover region where binding has been reported; open ellipses indicate regions of potential HSTF binding further upstream from the initiation site.

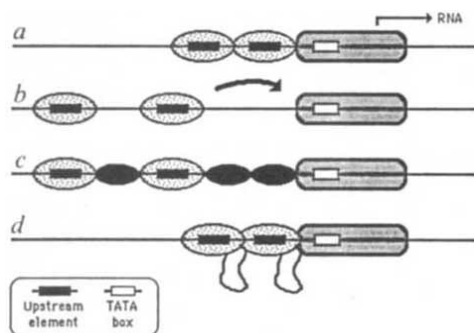


Fig. 5 Models for the action of promoter-specific factors. *a*, Transcription factors (stippled) bound to upstream elements, in direct contact with other proteins (shaded) bound at the initiation site; *b-d*, action at a distance. *b*, Upstream factor shifts position to contact proteins at initiation site, or alternatively, alters conformation of DNA, with effect felt at initiation site. *c*, Additional proteins bind interstices to create a continuous scaffold of protein in the promoter region. *d*, Transcription factors condense into a compact nucleoprotein structure, with looping-out of intervening DNA.

to explain, however, because despite its marked sequence asymmetry, the region is clearly involved in the genesis of transcripts from both viral strands^{14,43-45,47-51} and retains its ability to direct early RNA synthesis when inverted⁴⁶. This apparent paradox has not been resolved, although a simple possibility that cannot yet be excluded is that activation of transcription in opposite directions occurs by different and independent mechanisms.

Activation of transcription by enhancers

One persistent question in the field is whether enhancers are fundamentally different from upstream promoter elements of the sort that interact with Sp1 or HSTF. Clearly, as more experiments have been done, many upstream promoter elements have been found to have characteristics once thought to be distinctive properties of enhancers, such as bidirectionality and a degree of flexibility in spacing^{42,46,81,84}. So far, however, only enhancers have shown an ability to influence transcription over distances of a kilobase or more, and to work upstream or downstream of the promoter.

It seems probable that enhancers, like promoters, will turn out to be regions of interaction for site-specific DNA binding proteins. This has been shown directly for the enhancer-like glucocorticoid response elements of murine mammary tumour virus and the human metallothionein II_A gene, both of which bind glucocorticoid receptor^{5,85-87}. It is uncertain whether enhancers are sites for binding of a single protein or, like promoters themselves, are sites for assembly of a multiprotein complex, with some proteins binding directly to the DNA and others involved in other aspects of the reaction. There is evidence that different enhancers compete with each other for limiting components in the cell^{87,88}, which is consistent with the idea that various cellular and viral enhancers interact with at least some common factors.

Several groups have been successful in showing an effect of the SV40 enhancer in an *in vitro* system where transcription is activated 10-fold or more under optimal conditions⁸⁹⁻⁹². It is not yet certain, however, that the activation is caused by the enhancer *per se*, and not to other sequences, such as elements of the late promoter, that are present in the same region of SV40. In addition, the unique and distinguishing feature of enhancer activity, the stimulation of promoters over distances of ≥ 1 kilobase (kb), and from downstream of the gene, has not yet been demonstrated with an *in vitro* system.

Little more is known now about how enhancers transmit their effects over distances than when these elements were first discovered. Assuming that enhancers act at the level of initiation of transcription^{93,94}, it seems evident that they must facilitate some rate-limiting step; moreover, if they are binding sites for proteins they should follow the ordinary rules governing pro-

tein-DNA interactions. The models discussed previously for upstream promoter elements are therefore likely to be applicable to enhancers as well. A looping out of intervening DNA, in particular, could explain several features of enhancer-promoter interactions⁸³, and would allow a DNA-binding protein to exert its effect over any distance.

Conclusions

In this review we have chosen to focus on experiments involving biochemical fractionation and analysis of proteins from cell extracts. To keep this review brief, we have largely omitted discussion of several valuable alternative approaches. These include the use of enzymatic and chemical probes for analysis of chromatin structure in promoter and enhancer regions, as well as attempts to obtain mutants in the genes encoding trans-acting factors. Similarly, we have not discussed trans-acting factors such as the adenovirus E1A protein, which seem to modulate transcription by a mechanism other than sequence-specific DNA binding.

Although many details remain to be established, recent work clearly indicates the existence of promoter-specific transcription factors that recognize distinctive sequence elements found 40-100 bp upstream from the initiation site in eukaryotic protein-coding genes. Several such factors have been isolated and each factor recognizes a different sequence element. Binding activates transcription and allows the transcription machinery to discriminate between different promoters in an *in vitro* reaction. Within a promoter there are often multiple binding sites for the same or different factors, and the distance to the start site of transcription can vary significantly, suggesting that a mechanism exists for transmitting the activating signal through at least short stretches of intervening DNA. In addition to the sequence-specific DNA binding proteins, several other factors required for transcriptional initiation have been partially characterized, and these proteins, together with RNA polymerase, assemble via an ordered pathway into a functional preinitiation complex.

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ARTICLES

Residence time of thorium, uranium and lead in the mantle with implications for mantle convection

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The residence times of thorium, uranium and lead in the upper mantle are estimated at ≤ 600 Myr, and should be similar for related elements that are strongly concentrated in the continental lithosphere. Their upper mantle abundances are close to steady state and are mostly supported by entrainment of material from a lower mantle layer with long-term convective isolation. These lithophile trace elements and rare gases, such as ^3He , are effective chemical probes of the deeper mantle.

ONE of the main aims of geochemistry is to quantify the mass transfer processes that have occurred between different portions of the Earth, such as those responsible for the generation of the continental crust and atmosphere. The development of the continents, for example, has resulted in a marked transfer of the principal heat-producing elements (K, U and Th) and related large-ionic-radius trace elements from the mantle to the crust. A limit on the proportion of mantle involved in forming the continents has been imposed from the mass balance of those parent and daughter atoms involving the radiogenic isotopes of Nd, Sr, Hf and Pb (refs 1–4). These geochemical considerations together with those of the primordial and radiogenic components of the rare gases⁵ have been central to arguments as to whether convection extends throughout the whole mantle or is separated into two layers. In the latter case of layered convection, attention has been drawn to the requirement for transport of at least heat and He across the separating boundary layer⁶.

The depleted character of the upper mantle is evident in both the elemental and isotope compositions of mid-ocean ridge basalts (MORB). In particular, trace elements that strongly partition into silicate melts (bulk solid/liquid distribution coefficient $D_i \ll 1.00$), such as Cs, Rb, K, Th, U and light rare earths, are estimated to be present at $\leq 20\%$ of their initial

abundances^{1–4}. This depleted character is evident not only in Recent MORB and Phanerozoic ophiolites but also in some Archaean komatiites and basalts^{7,8}, indicating that part of the mantle has been depleted for $\sim 3,000$ Myr. Because elements with the lowest D_i values are extracted more efficiently than those with larger D_i during melting, it can be anticipated that some relationship exists between transport, distribution coefficient (D_i), residence time ($\bar{t}_i$) and degree of mantle depletion.

In this article, we suggest that limits can be placed on the residence times of Pb, Th and U in the mantle, and that the upper mantle has close to steady-state concentrations for these elements. The results have important implications for the origin of chemical and isotope heterogeneity in the mantle, the degree of boundary-layer interaction in a convectively-stratified mantle, and the early differentiation of the terrestrial planets.

Residence times

Th and U are highly incompatible elements that are strongly depleted in the Earth's upper mantle. The Th/U ratio or κ value ($^{232}\text{Th}/^{238}\text{U}$ ratio) of the bulk Earth has been estimated at ~ 3.85 from the Pb isotope compositions of conformable lead ore deposits^{9,10}, in good agreement with the value of 3.9 ± 0.1

Table 1 Th/U ratios in oceanic basalts from Pb and Th isotopes

	$\bar{\kappa}_{\text{Pb}} \pm 1\sigma$	n	Ref.	$\kappa_{\text{Th}} \pm 1\sigma$	n	Ref.
MORB						
Mid-Atlantic Ridge	3.78 ± 0.07	71	17, 24, 25, 36, 41, 42	2.5	—	43
East Pacific Rise	3.73 ± 0.06	31	17, 24, 25, 41, 42	2.5 ± 0.2	18	14
Mid-Indian Ridge	3.89 ± 0.11	21	17, 24, 25, 44			
Juan de Fuca-Gorda	3.73 ± 0.04	24	45			
Seamounts						
North-east Pacific	3.73 ± 0.03	18	45			
Islands						
Hawaii	3.84 ± 0.04	55	11, 17, 46, 47	3.0 ± 0.2	18	48
Iceland	3.81 ± 0.03	39	25, 49, 50	3.0 ± 0.2	17	51
St Helena	3.75 ± 0.02	14	17, 52			
Aurolas	3.75	5	53			
Ascension	3.81 ± 0.02	16	17, 52, 54			
Azores	3.84	5	17	2.6	4	55
Bouvet	3.84	3	17			
Canaries	3.92 ± 0.03	40	17			
Marquesas	3.98 ± 0.04	10	53			
Guadalupe	3.99	4	17			
Tristan de Cunha	4.17	8	17, 52	3.7	5	55
Gough	4.20	8	17			
Kerguelen	4.24 ± 0.12	24	56			
Walvis Ridge	4.25	9	57			

n = number of samples. $\bar{\kappa}_{\text{Pb}} = \{^{208}\text{Pb}^*/^{206}\text{Pb}^*\}(\text{e}^{\lambda^{238}\text{T}} - 1)/(\text{e}^{\lambda^{232}\text{T}} - 1)$ where T is the age of the Earth (4,550 Myr), λ_i the decay constant of parent isotope i , and $^{208}\text{Pb}^*/^{206}\text{Pb}^*$ is corrected for primordial Pb (ref. 16). $\kappa_{\text{Th}} = (\lambda_{238}/\lambda_{232})(^{232}\text{Th}/^{230}\text{Th})$ where $(^{232}\text{Th}/^{230}\text{Th})$ is an activity ratio.

deduced from igneous rocks of various ages^{11,12}. The latter value of 3.9 is adopted here for the bulk Earth Th/U ratio, but a precise value is not critical to the central argument.

The present-day Th/U ratio of the upper mantle may be estimated from Th and U abundances or from $(^{232}\text{Th}/^{230}\text{Th})$ activity ratios of MORB, derived by melting the mantle beneath spreading ridge axes. Measured $(^{232}\text{Th}/^{230}\text{Th})$ activity ratios from the East Pacific Rise and other MORB (Table 1) correspond to Th/U = 2.5 in their mantle sources, however, the observation that $1 < (^{230}\text{Th}/^{238}\text{U}) < 1.3$ for most MORB also suggest that Th enters the melt phase preferentially over U and that $D_{\text{Th}} < D_{\text{U}}$ (refs 13, 14). This is consistent with Th/U concentration ratios reported by Jochum *et al.*¹⁵ which range from Th/U = 1.5 to 3.1 as the Th content increases, and should, therefore, be an upper limit on the Th/U ratios of their sources. When taken together, this implies that Th/U $\leq$ 2.5 in the upper mantle sampled at spreading ridges, or around half of any estimate for the bulk Earth.

An additional estimate of Th/U, albeit a time-integrated average over Earth history, can be calculated from the Pb isotope compositions of oceanic basalts; values of $\bar{\kappa}_{\text{Pb}}$ are derived from the radiogenic additions to ^{208}Pb and ^{206}Pb from their respective parent isotopes ^{232}Th and ^{238}U making allowance for primordial Pb (ref. 16) (Table 1). Most MORB show a restricted range from 3.65 to 3.89 with an average value of ~ 3.75 (Table 1); ocean-island basalts exhibit a larger range ($3.7 < \bar{\kappa}_{\text{Pb}} < 4.2$). Thus, the Th/U ratio that the Pb in oceanic basalts has 'seen' over its history ($\bar{\kappa}_{\text{Pb}}$) is higher than the deduced Th/U < 2.5 of the upper mantle, but close to the bulk Earth value of ~ 3.9 .

At first sight, these relationships^{11,17,18} appear paradoxical because the low Th/U ratio of MORB, symptomatic of depletion, is expected to be a long-standing feature of the upper mantle. In Fig. 1, the response of the $\bar{\kappa}_{\text{Pb}}$ parameter (and $^{208}\text{Pb}^*/^{206}\text{Pb}^*$) to recent U-Th-Pb fractionations is examined in a simple two-stage model reducing the Th/U ratio from 3.9 to 2.5 at various times in the past. For a range of possible μ values ($^{238}\text{U}/^{204}\text{Pb}$), the Pb now sampled by MORB could not have been in an environment with Th/U = 2.5 for more than 600 ± 200 Myr.

The simplest explanation for this observation is that the residence time ($\bar{t}_{\text{Pb}}$) of Pb in the upper mantle is short (~ 600 Myr), and, therefore, 'buffered' by the introduction of Pb into this portion of the mantle from another reservoir possessing an approximately bulk Earth Th/U = 3.9. Such an effect is easier

to sustain if the concentration of Pb in the upper mantle is very low compared with the introduced material. The steady-state residence time ($\bar{t}_i$) for an element (i) is given by $\bar{t}_i = [c_i/c_0]/\dot{m}_i$ where c_i is the steady-state concentration in, and c_0 that of, material added to the reservoir at a mass flux of $\dot{m}_i$ relative to the total mass of the reservoir, so that $\bar{t}_{\text{Th}}/\bar{t}_{\text{U}} = [c_i/c_0]_{\text{Th}}/[c_i/c_0]_{\text{U}}$. For a steady-state Th/U ratio ($[c_i]_{\text{Th}}/[c_i]_{\text{U}}$) of 2.5 and a bulk Earth value of 3.9 for the input ratio ($[c_0]_{\text{Th}}/[c_0]_{\text{U}}$), $\bar{t}_{\text{Th}}/\bar{t}_{\text{U}} = 0.64$. Similarly, if it is assumed, for example, that the $^{238}\text{U}/^{204}\text{Pb}$ ratio of the steady-state upper mantle (μ_1) is 6.0, that of the input material (μ_0) is 8.0 and fixing $\bar{t}_{\text{Pb}} = 600$ Myr, then it follows that $\bar{t}_{\text{U}} = 450$ and $\bar{t}_{\text{Th}} = 290$ Myr. The Pb isotope compositions and Th/U ratios can be readily understood in terms of short residence times for these three elements in the upper mantle, but with $\bar{t}_{\text{Th}} < \bar{t}_{\text{U}} < \bar{t}_{\text{Pb}}$ in the same order as their respective D_i .

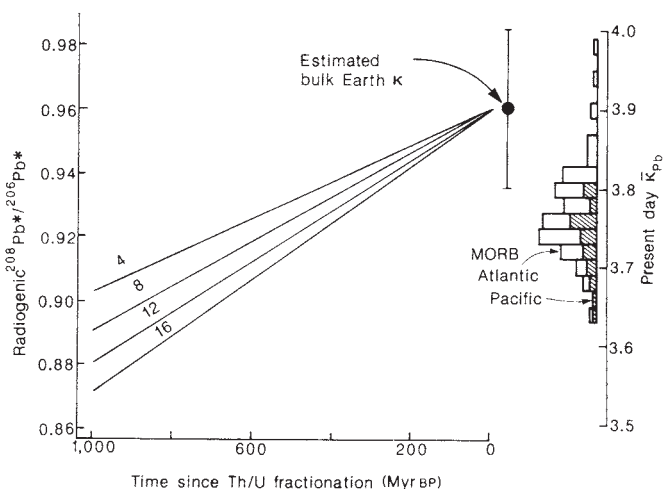


Fig. 1 Evolution of radiogenic $^{208}\text{Pb}^*$ and $^{206}\text{Pb}^*$ with time in a system with Th/U = 2.5 assuming a starting time-integrated Th/U ($\bar{\kappa}_{\text{Pb}}$) of 3.9. Lines are constructed for $^{238}\text{U}/^{204}\text{Pb}$ (μ) values ranging from 4 to 16. Comparison with the observed range of $\bar{\kappa}_{\text{Pb}}$ values (and $^{208}\text{Pb}^*/^{206}\text{Pb}^*$) of mid-ocean ridge basalts (MORB) indicates that they have spent 600 ± 200 Myr in a low Th/U environment. $\bar{\kappa}_{\text{Pb}}$ is defined in Table 1.

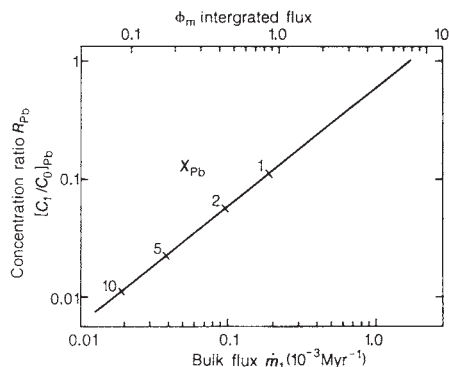


Fig. 2 The relationship between the steady-state bulk entrainment flux into the upper mantle and the degree of Pb depletion. ϕ_m is the integrated bulk flux over 4,550 Myr assuming a constant bulk flux $\dot{m}_1$, and are both expressed as a fraction of the upper mantle mass. c_1 is the steady-state upper mantle concentration and c_0 that of the entrained material, so that $[c_1/c_0]_{Pb}$ (R_{Pb}) is the Pb depletion factor for the upper mantle. Some restriction on R_{Pb} and hence $\dot{m}_1$ and ϕ_m arise from the Pb content of MORB. A value of $X_{Pb} = 1$ corresponds to the R_{Pb} ratio inferred to provide 400 p.p.b. Pb in MORB, assuming that major and trace elements are contributed to MORB from equal mantle source volumes and taking $[c_0]_{Pb}$ as that in the bulk silicate Earth⁵⁸. It is possible that Pb is contributed from a volume X_{Pb} times larger than that giving rise to the major elements in the oceanic crust. Acceptable solutions are likely to be restricted to $1 < X_{Pb} < 3$, corresponding to $0.25 < \phi_m < 0.5$.

Buffering and entrainment

The short residence time implied for Pb necessitates the Pb in the mantle region now sampled in MORB to have been introduced relatively recently from some other reservoir and therefore have a similar Pb isotope signature. Candidates for this external reservoir must be some portion of the continental lithosphere or a lower-mantle region which becomes entrained at its boundary with the convecting upper mantle. The first possibility is the upper continental crust, which is a substantial reservoir for Pb and either directly¹⁹, or as a component of pelagic sediment, might be injected into the mantle. Crustal Pb has a higher $^{207}\text{Pb}/^{204}\text{Pb}$ ratio than MORB that has been used to identify subducted sedimentary contributions to island-arc volcanic suites²⁰. As this elevated $^{207}\text{Pb}/^{204}\text{Pb}$ ratio is predominantly the result of ancient U/Pb fractionation recorded by the decay of now largely extinct ^{235}U , this difference indicates that the bulk of MORB Pb does not come from this source. Moreover, this situation is likely to have pertained throughout the time required to generate such a $^{207}\text{Pb}/^{204}\text{Pb}$ shift ($>10^9$ yr). An upper crustal source, and thus suggestions of substantial recycling of crustal Pb (and presumably other highly incompatible elements) back into the mantle^{21,22} are largely eliminated, although early recycling cannot be excluded. Furthermore, the subduction zone environment apparently efficiently filters continental-derived Pb, and probably also other constituents.

The second reservoir that might feasibly buffer upper mantle Pb is the mantle part of the continental lithosphere²³. This possibility may be best considered with respect to the magnitude of the bulk flux into the upper mantle that is required when integrated over a substantial portion of Earth history; a quantity that is minimized if the concentration of Pb in the upper mantle is very small compared with that in the bulk material added to the reservoir. This 'trade-off' is investigated in Fig. 2, where the concentration ratio R_i is the steady-state concentration of Pb in the upper mantle expressed as a remaining fraction of the input concentration $[c_1/c_0]_{Pb}$ for $\bar{t}_{Pb} = 600$ Myr, and the total integrated bulk flux (over 4,550 Myr) into the reservoir (ϕ_m) is relative to the mass of the upper mantle.

Some limit on the Pb concentration in the upper mantle may be fixed from a consideration of that in MORB. Assuming that MORB represent a 10–20% partial melt followed by ~40% fractional crystallization, then for an average erupted value of

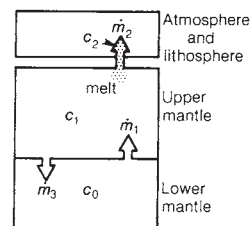


Fig. 3 A simple box model involving limited bulk entrainment at the interface between an upper and lower mantle region which are otherwise convectively isolated. It is assumed that the mass of the upper mantle box has remained constant over Earth's history, although models involving an increase in this mass and no return flux to the lower mantle box are equally viable. The concentrations of Th, U and Pb in the upper mantle box are assumed to be at steady state and supported by entrainment from the lower, minimally depleted box. The complex fractionation and removal of these elements into the continental lithosphere plus atmosphere is simply described by the single-stage extraction of partial melt as indicated. The differential partitioning of elements into the melt at this point results in variable residence times of elements in the upper mantle box and the steady-state element ratios (such as Th/U) to be different from the ratios in the material entrained at the lower boundary. However, at steady state the element ratios in input and output are equal.

400 parts per 10^9 Pb (refs 17, 24, 25), the source would contain around 20–30 parts per 10^9 . This value may be reduced by a factor X_{Pb} if the oceanic crust samples Pb from a volume that is X_{Pb} larger than from which it extracts major elements^{26,27}. This limit can now be compared with an estimate of the Pb available in the mantle part of the continental lithosphere which probably constitutes $\leq 10\%$ of the upper mantle. Comparatively Pb-rich ultramafic xenoliths from this region contain ~100 parts per 10^9 Pb (ref. 28), only a factor of 10 higher than this upper mantle value (that is, $[c_0/c_1]_{Pb} = 10$). These considerations, therefore, lead to the conclusion that the lithosphere mantle must exchange completely with the convecting upper mantle every few hundred million years to buffer upper mantle Pb, and would have a comparable age. This is, however, inconsistent with the longevity ($>10^9$ yr) of the lithosphere mantle outside the main convective flow documented by the isotope systematics of xenoliths²⁸ and silicate inclusions in diamonds²⁹. Although this does not preclude the possibility that either the mantle or crustal portions of the continental lithosphere make a contribution, they seem not to be the predominant source.

The only viable source remaining for the bulk of this Pb seems to be the lower mantle, which could buffer upper-mantle Pb for the whole of Earth's history without invoking a Pb content exceeding the bulk Earth value (Fig. 2) by virtue of its large mass. If normal ridges strip a much larger volume of mantle of incompatible elements than is indicated by the volume of oceanic crust alone^{26,27} then the estimated concentration of Pb in the upper mantle would be lowered proportionally, and similarly the flux across the boundary required to sustain this Pb. For instance, if this additional volume amounted to between 1 and 3 times that required to support the major elements in the oceanic crust ($1 < X_{Pb} < 3$), it would follow from Fig. 2 that the upper mantle would have been exchanged between a quarter and half by mass over geological time with the lower reservoir. This value is more than a factor of 10 greater than proposed by Olson³⁰ for the exchange across this boundary from scaled convection experiments.

Steady-state upper mantle

The concept of a steady-state upper mantle requires that the input and output fluxes are balanced. This has been used previously⁶ for ^3He , on the assumption that the flux into the oceans from ridges will balance an entrainment flux into the upper mantle from below. The suggested behaviour of Pb implies that, in addition to the rare gases, other highly incompatible elements in the upper mantle may also have reached steady-state con-

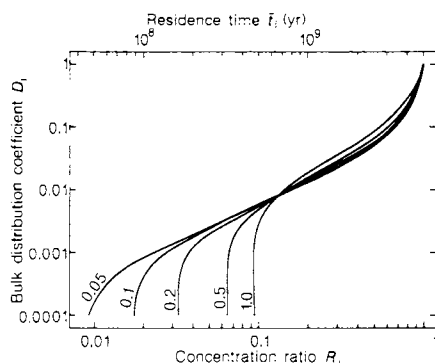


Fig. 4 Comparison of bulk distribution coefficient (D_i) and residence time ($\bar{t}_i$) in the upper mantle portion of the model depicted in Fig. 3. The ratio of steady state to input concentration (R_i) is related to the residence time by $\bar{t}_i = R_i / \dot{m}_1$ (see text). Curves are shown for various melt fractions (F , %) corresponding to the output material, but fixing $\dot{m}_1 = 0.0002 \text{ Myr}^{-1}$, $D_{pb} = 0.007$ and $\bar{t}_{pb} = 600 \text{ Myr}$. To fractionate elements of low D_i , such as Th and U, F must be small and of the order of D_i . Where $\bar{t}_i$ approaches the age of the Earth, elements will not have achieved a steady state. Elements of lowest D_i may be present in the steady state in proportions similar or identical to the input material depending on F .

centrations. Some of the ramifications of buffering by entrainment of lower mantle material are best explored within the context of a very simple model (Fig. 3) which uses several simplifying assumptions: (1) material from the lower mantle enters the upper mantle by bulk entrainment at their mutual interface without element fractionation; (2) incompatible elements in the upper mantle are transported into the overlying lithosphere and atmosphere which, as a first step, involves the generation of a small fraction of partial melt, causing element fractionations that can be modelled as Rayleigh melting; (3) the mass of the upper mantle has remained constant, with a return flux to the lower mantle during entrainment; (4) the lower mantle can be treated as an infinite reservoir with primordial mantle (bulk Earth minus core) trace-element abundances; (5) the system has reached a steady state. Note, however, that scenarios involving a variable upper mantle mass with time¹⁻⁴ have not been explored here but are equally compatible with the geochemical evidence.

The steady-state concentration $[c_1]_i$ of an element of interest in the upper mantle compared with its input value $[c_0]_i$ can be shown to be

$$R_i = [c_1 / c_0]_i = 1 / \{1 + (\dot{m}_2 / \dot{m}_1)[f_i - 1]\}$$

and its residence time is

$$\bar{t}_i = R_i / \dot{m}_1$$

where $\dot{m}_1$ and $\dot{m}_2$ are the bulk mass fluxes shown in Fig. 3 expressed relative to the total mass of the upper mantle. f_i is an enrichment factor relating $[c_2]_i$ to $[c_1]_i$ during partial fusion³¹

$$f_i = [c_2 / c_1]_i = \{1 - [1 - F]^{1/D_i}\} / F$$

where D_i is the bulk distribution coefficient during melting and F is the melt fraction involved. The melt fraction F describes in a simple manner the complex extraction of elements from the mantle into the continental lithosphere. To produce the observed element fractionations F must be $\approx D_i$. An important parameter for the upper mantle is the integrated bulk flux across its lower boundary over geological time (ϕ_m). This is readily obtainable, as $\dot{m}_1$ in the steady state is $R_i / \bar{t}_i$ which, using $R_{pb} = 0.06$ (from Fig. 2) and $\bar{t}_{pb} = 600 \text{ Myr}$, implies $\dot{m}_1 \approx 0.0001 \text{ Myr}^{-1}$, or roughly half of the mass of the upper mantle has exchanged with the lower over geological time ($\phi_m \approx 0.5$). This value will be smaller if R_{pb} is smaller (for instance, if $X_{pb} > 1$).

A processing timescale (T_p), defined as the time taken to extract

partial melt once from the upper mantle mass, is given by

$$T_p = F / \dot{m}_2 = f_i \bar{t}_i / \{1 - R_i\}$$

which is also the residence time of a perfectly incompatible element ($D_i = 0$). T_p is related to the mixing time (T_m) of the upper mantle down to a scale sampled by a basalt, but not in a straightforward manner, and is estimated at $< 600 \text{ Myr}$ as $T_m < T_p \leq \bar{t}_{pb}$. An exchange timescale (T_e) can also be defined, and is the time taken to exchange the upper mantle mass once, which is simply $1 / \dot{m}_1$.

The behaviour of the system is illustrated in Fig. 4, where the concentration ratio R_i and the residence time $\bar{t}_i$ are shown as a function of D_i for various values of F , fixing $\dot{m}_1 = 0.0002 \text{ Myr}^{-1}$, $\bar{t}_{pb} = 600 \text{ Myr}$ and $D_{pb} = 0.007$. The D_i / F ratio divides elements into three groups with contrasting behaviour. First, for $D_i / F \gg 1$, $\bar{t}_i >$ the age of the Earth and so a steady-state R_i does not pertain; second, for $D_i / F \approx 1$, $\bar{t}_i$ and R_i are at steady state and depend strongly on D_i but not F ; and third, for $D_i / F < 1$, constant $\bar{t}_i = T_p$ and $R_i \approx T_p \dot{m}_1$ (independent of D_i) result from the supply of material being rate limiting. For the time-dependent (non-steady state) case it can be shown that the concentration ratio decays exponentially to the steady-state value R_i in a characteristic time equal to $\bar{t}_i$.

Consideration of this simple model suggests that the more compatible elements ($D_i > 0.1$), for example, the heavy rare-earth elements (HREE), are unlikely to have reached steady-state concentrations in the upper mantle. Furthermore, the highly incompatible elements may be uniformly extracted over the upper mantle processing time (T_p) consistent with the observation that the abundance ratios of Cs, Rb and Ba (all with $D_i < 0.001$) are nearly constant in MORB³².

For those elements at steady state, input and output fluxes are necessarily equal, for example, the Th/U ratio of both the input and output materials should be 3.9 even though the steady-state ratio is 2.5. The material that is extracted from the upper mantle to reside ultimately in the continental lithosphere must be characterized by a Th/U ratio of 3.9 that should, in principle, identify the egress route. The Th/U ratios measured in MORB glasses¹⁵ are mostly < 3.0 , and tholeiitic series basalts from oceanic island arcs are commonly even lower³³; the fractionation of Th/U required does not seem to occur in either of these environments. This is analogous to the problem of the site of the $\sim 40\%$ reduction in the Sm/Nd ratio that accompanies the production of continental crust and should be addressed in any model of mantle chemistry, whether steady state or otherwise. Only calc-alkaline series island arc volcanics have Th/U and Sm/Nd ratios³³ in the range of continental crustal materials, and the problem of Th-U and rare-earth fractionation may be linked to their genesis. The present production rate of oceanic island arc volcanics³⁴ and their abundances of highly incompatible elements³³ appear comparable with that required to balance the fluxes of these elements into the upper mantle in the steady state.

Mantle chemistry and convection

The isotope compositions and abundances of the incompatible trace elements in mantle-derived materials have been exploited to constrain theories of basalt petrogenesis, delimit the major geochemical reservoirs and their interactions and appraise the possibilities of layered versus whole-mantle convection. The observation that those trace elements with particularly small distribution coefficients ($D_i \ll 1$) have $\bar{t}_i \approx 600 \text{ Myr}$, and that $\bar{t}_i$ varies with D_i , has important implications for the interpretation of element and isotope abundances in mantle-derived melts.

Correlations between the radiogenic isotopes of Sr, Nd, Hf, Pb and the rare gases have been a focal point of mantle geochemistry. It is now clear that only in those instances where the residence times of the isotopes of interest are the same (such as for two of the three Pb isotope systems) is their relationship likely to be straightforward. Where $\bar{t}_i$ values differ, as between He and Sr, a large degree of decoupling of their isotope compositions is to be expected, and this is exactly what is observed³⁵.

The significance of the well-documented correlations between Pb and Sr isotopes in MORB^{24,36}, and the Nd and Sr isotopes in oceanic basalts as a whole³⁷, should be re-evaluated in view of the available constraints on their residence times.

The relationships between D_i and steady-state concentration require that the layering in the mantle, as reflected in the concentration gradients across the boundary, would be most evident for those elements with the lowest D_i (such as Pb, Th, U, K, He and Ar) and progressively less perceptible as D_i increases to 1.0, whereas the opposite would be true for isotope gradients. Furthermore, that the lower mantle reservoir is mainly responsible for the supply of short t_i elements to the upper mantle must imply that they have been retained within this layer for the whole of Earth's history. The observation that most MORB have Pb isotopic compositions not far removed from that implied for a simple closed system over the age of the Earth (the geochron) is easily explained on this basis.

The small dispersion of κ_{Th} in MORB of 2.5 ± 0.2 (Table 1) when compared with the assumed bulk Earth input value of 3.9 indicates that the introduced Th has become exceedingly well-mixed into the upper mantle by the time that such Th is sampled at spreading ridge axes. The time required to mix the length-scale of input material down to the scale of sampling at ridges is, therefore, less than the residence time of Th in the system, that is a few hundred million years, and agrees with estimates from convection experiments in a layered mantle^{38,39}. Moreover, the islands such as Hawaii and Iceland that are considered, on the basis of high $^3He/^4He$ ratios, to lie over deep seated plume structures in the mantle have $\kappa_{Th} = 3.0 \pm 0.2$ indicating that entrained material is not exclusively sampled at these islands, but a considerable portion of depleted mantle is also included.

The implied short t_i values for the heat-producing elements K, Th and U has important consequences for the early thermal history of the Earth. It can be expected that these elements were rapidly concentrated, if not stabilized, in the outermost portion of the Earth (like the Moon) within the first few hundred million years of the planet's history; a significant fraction of the Earth's radiogenic heat production would, as a result, have been highly localized from earliest times. This partitioning of highly incompatible elements may have been unrelated to the formation of continental crust, a phenomenon that essentially involves major elements, and the fractionation history of Th/Pb and U/Pb recorded by the isotope structure in the continents may conceivably predate the formation of continental crust *per se*. In the case of the Moon, the large concentrations of incompatible elements in components of the lunar crust⁴⁰ do not necessarily imply that there was accompanying large-scale major element differentiation of the interior.

There arises an additional consequence for the geochemical mass balances of the abundances and isotopes of Sr and Nd between the crust and upper mantle¹⁻⁴, that has been used to determine the proportion of mantle depleted in forming the continental crust. These do not directly correspond to the proportions of a convectively isolated layer if material has been exchanged with another reservoir, and will also vary between elements in accordance with their respective residence times. However, the estimated entrainment of ~ 0.1 part of the upper mantle per 10^9 yr will not invalidate the general conclusions arrived at. Furthermore, the addition of highly incompatible elements to the continents will be controlled by the entrainment process and will proceed at a lower rate than their maximum rate of incorporation in the past.

Conclusions

The demonstration that incompatible trace elements with low D_i values may have residence times as low as a few hundred million years in the upper mantle has provided new insights into various questions concerning mantle convection, melting and the early differentiation history of the Earth. The very short residence time of lead ($t_{Pb} \sim 600$ Myr) and related elements requires substantial buffering from a lower mantle source, which is only feasible if the upper mantle and lower mantle convect as separate layers and entrainment of lower mantle material is significant (amounting to approximately half of the upper mantle mass over geological time). Recycling of incompatible trace elements from the upper continental crust into the mantle can have only limited importance; hence, the subduction zone environment must act as an effective barrier to the passage of these constituents. The lower mantle must still retain volatiles (3He , for example) and a complement of incompatible elements, indicating that the differentiation history of the planet has not included an appreciable purging of these elements from the lower mantle as it has the upper. Finally, elements of differing bulk distribution coefficient will 'probe' the deep interior of the Earth to differing extents. The most incompatible elements in spreading ridge basalts, 3He , Th, U and Pb for instance, seem to be dominated by the entrainment of lower mantle material and provide a window through to the deeper mantle and an insight into the chemistry of that region. The budgets of the more compatible elements, however, are still dominated by their initial upper mantle inventories.

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Diversity and structure of genes of the α family of mouse T-cell antigen receptor

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We have analysed 19 complementary DNA clones encoding the α -chain of the T-cell antigen receptor derived from thymic transcripts, and find that 15 of them contain partial or complete variable (V_α) genes. Seven of these genes cross-hybridize to over 40 germline V_α gene segments in Southern blot analyses. Of the 19 joining (J_α) sequences examined, 18 seem to be encoded by distinct gene segments, hence the repertoire of J_α gene segments is much larger than those of the immunoglobulin or T-cell receptor β -chain gene families. We suggest that the variable domains of immunoglobulins and T-cell antigen receptors are similar in structure.

THE antigen-specific receptors on T cells, like the immunoglobulin molecules used as antigen-specific receptors on B cells, can recognize and bind an almost unlimited range of antigenic determinants. T-cell antigen recognition differs from immunoglobulin antigen recognition in that it occurs only in the context of another cell-surface protein encoded by the major histocompatibility complex (MHC), a property termed MHC restriction (for review, see ref. 1). This unique property raises the question of whether the structures of T-cell antigen receptors fundamentally differ from those of immunoglobulins.

The T-cell receptor is a disulphide-bridged heterodimer composed of α - and β -chains, each divided into variable (V) and constant (C) regions²⁻⁴. Studies of the structure and organization of β -chain gene elements have revealed that the V region of the β -chain, like that of the immunoglobulin heavy (H) chain, is encoded by three separate germline gene segments, V_β , diversity (D_β) and joining (J_β), that rearrange during T-cell differentiation to form a functional V_β gene^{5,6}. There are two closely linked C_β genes, $C_\beta 1$ and $C_\beta 2$, each associated with a cluster of six functional J_β gene segments^{5,7,8}. Each J_β cluster has one D_β gene segment located ~500-700 nucleotides upstream^{9,10}.

T and B cells use several mechanisms to generate diversity in the antigen-binding V region: (1) germline diversity. Both T and B cells use many different germline V, D and J gene segments to form a V gene. (2) Combinatorial joining. Each D gene segment seems capable of joining to any downstream J gene segment, and any V may join to any D-J combination^{11,12}. (3) Somatic mutation. The V_β genes share two somatic mutational mechanisms with their immunoglobulin counterparts. First, there is flexibility in the sites at which the gene segments are joined and this generates diversity in the junctional regions^{9,10}. Second, random nucleotides may be added to either side of the D gene segments during its joining to the V and J gene segments^{9,10}, a mechanism denoted N-region diversification¹³. Immunoglobulin V-region genes may undergo somatic hypermutation at a late stage of B-cell development, a phenomenon that has not been seen in most V_β genes^{5,11,12,14}.

The V_β gene segments differ strikingly from their immunoglobulin κ and H-chain homologues in subfamily organization. A subfamily is defined as those V gene segments that are 75% or more similar to one another at the nucleic acid level and hence cross-hybridize in Southern blots under stringent hybridization conditions¹⁵. The immunoglobulin V subfamilies are composed of 4-50 or more members¹⁵⁻¹⁷. In contrast, the known mouse V_β gene segments represent eight subfamilies, six with only one member, one with two members, and one with three members^{11,12}.

Recently, mouse cDNA clones of α -chain transcripts have been described^{18,19}. The α -chains seem to consist of V, J and C regions. Here we present the sequences of 19 additional α -cDNA clones. Analyses of these 21 α -cDNA sequences reveal that: (1) the V_α gene segments are derived from at least 10 subfamilies

each containing 1-10 members; (2) there are many J_α gene segments; (3) the diversification mechanisms for V_α genes are similar to those seen for β -chain and immunoglobulin genes; and (4) the general structure of the α -polypeptides resembles that of immunoglobulin and β -chains in primary and secondary structural characteristics.

Sequences of α -cDNA clones

Nineteen cDNA clones were isolated from a thymic library of inbred C57BL/Ka mice¹¹ using a C_α probe²⁰. The cDNA inserts were subcloned into the M13mp18 bacteriophage vector and the sequences determined using the specific-primer-directed dideoxynucleotide sequencing technique (Fig. 1)²¹. Four of the 19 α -cDNA clones (TA38, TA91, TA45 and TA20) do not have a recognizable V_α gene segment (see below); 17 contain J_α sequences; and 15 contain complete or partial V_α sequences (Figs 1, 2). The α -cDNA sequences in Fig. 1 are divided into leader, V, J and C regions according to their similarities to the V and J segments of immunoglobulins and β -chains. The V_α segment is ~90 residues long and has a leader of 19-22 amino acids (Fig. 1). The N-terminal amino acid of the mature V_α gene product is presumed to be 22 or 23 residues from the first conserved half-cysteine residue according to the general rules that have been deduced for determining the leader peptide cleavage site²². The 3' ends of the V_α sequences have been provisionally designated as the same length as that of a single germline gene segment, $V_\alpha 5H$, a member of the $V_\alpha 1$ subfamily, analysed by Winoto *et al.* in the accompanying paper²⁰. The 5' and 3' ends of four J sequences have been determined by sequence analysis of four germline J_α gene segments (TA84, TA19, TA65 and TA80)²⁰. The boundaries of the remaining J_α sequences were provisionally assigned by homology (see Fig. 1 legend). There are nucleotides present between the V_α and J_α sequences that might arise from various different sources (see ref. 20).

Subfamilies of V_α gene segments

The amino-acid sequences of the 15 V_α segments were aligned with one another and with two additional V_α segments from the literature, TT11 (ref. 18) and pHDS58 (ref. 19) (Fig. 2). The V_α segments can be divided into 10 different subfamilies, members of each subfamily being 75% or more homologous with one another at the DNA level. The V_α segments from different subfamilies are 20-54% similar to one another at the protein level (Table 1). This range of homologies is similar to that observed between the V_β and immunoglobulin V_H subfamilies¹¹. We have denoted these subfamilies as $V_\alpha 1$ to $V_\alpha 10$ (Fig. 2). Five of these subfamilies ($V_\alpha 1-5$) are represented by two members and five by only one.

To estimate the number of germline V_α gene segments in each subfamily, Southern blot analyses were performed on liver DNA from four different inbred strains of mice using V_α -containing

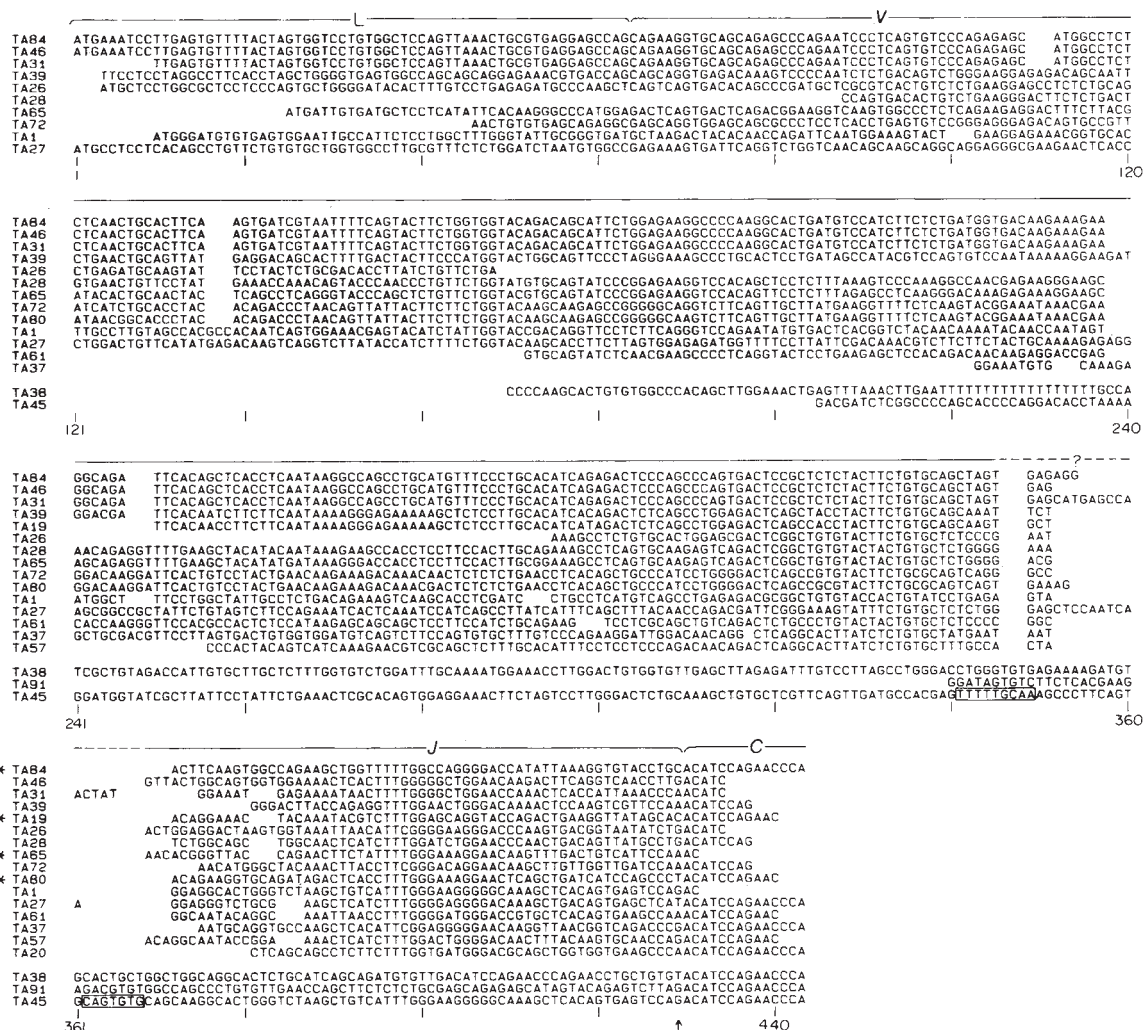


Fig. 1 Nucleotide sequences of 19 α -cDNA clones derived from the thymus. The leader (L), V, J and C regions are indicated. Clones TA38, TA91 and TA45 do not contain V_{α} sequences. The dotted lines between the V and J regions indicate junctional diversity arising from any one of several mechanisms (see ref. 20). The 3' ends of the V_{α} gene segments were arbitrarily chosen to be identical with the end of the germline V_{α} 5H gene segment²⁰. The 5' ends of the J_{α} gene segments were determined by comparison with the corresponding germline J_{α} gene segments (indicated by asterisks)²⁰ or by arbitrarily assuming that the J_{α} gene segment begins one codon after the end of the V_{α} gene segment. Assignments of the 5' ends of the J_{α} gene segments for the TA31 and TA27 cDNA clones are complicated by the presence of frameshift mutations at the junctional regions. Gaps are inserted to maximize similarity. The arrow at base 429 indicates diversity at the end of the J_{α} gene segment (see text). In clone TA45, the recognition sequences for DNA rearrangement are boxed. A 500-bp *Sau3A1* restriction fragment containing the C_{α} gene was used as a ³²P-labelled, nick-translated probe³⁷ to screen a λ gt10 cDNA library constructed from C57BL/Ka thymus messenger RNA (ref. 38, modified by Barth *et al.*¹¹). The cDNA inserts from C_{α} -containing clones were subcloned into the M13mp18 vector³⁹. The subclones were sequenced using the specific-primer-directed dideoxynucleotide chain termination method²¹. Synthetic oligonucleotide probes complementary to both strands of the C_{α} gene were used to select inserts of both orientations.

Table 1 Similarity of V_{α} sequences

	TT11	TA84	TA39	TA19	pHDS58	TA26	TA28	TA65	TA72	TA80	TA1	TA27	TA61	TA37	TA57
TT11	—	79	51	56	33	40	31	30	43	42	20	24	38	24	40
TA84	87	—	54	65	31	35	32	31	44	42	22	28	40	22	37
TA39	61	62	—	91	33	38	35	27	44	43	25	33	29	24	43
TA19	66	69	96	—	38	41	34	31	53	56	23	44	35	23	43
pHDS58	47	44	42	46	—	96	52	50	38	37	30	27	46	26	23
TA26	48	42	44	43	96	—	60	56	38	35	35	34	69	25	47
TA28	41	43	45	46	58	63	—	70	36	33	27	26	55	18	33
TA65	42	42	40	44	60	66	83	—	30	29	26	27	54	18	33
TA72	58	60	58	67	49	53	44	44	—	90	25	34	31	16	27
TA80	53	56	56	68	47	49	42	42	95	—	20	33	30	16	30
TA1	37	36	40	41	43	48	41	40	41	40	—	22	24	19	17
TA27	40	42	43	48	40	47	43	42	47	46	37	—	25	21	40
TA61	49	52	46	51	55	69	63	62	50	49	37	39	—	22	41
TA37	36	34	37	39	40	34	28	31	30	32	35	39	29	—	31
TA57	47	41	48	49	42	53	44	41	47	49	40	41	52	37	—

Numbers above the diagonal designate percentage similarity of protein sequences; those below the diagonal compare nucleotide sequences.

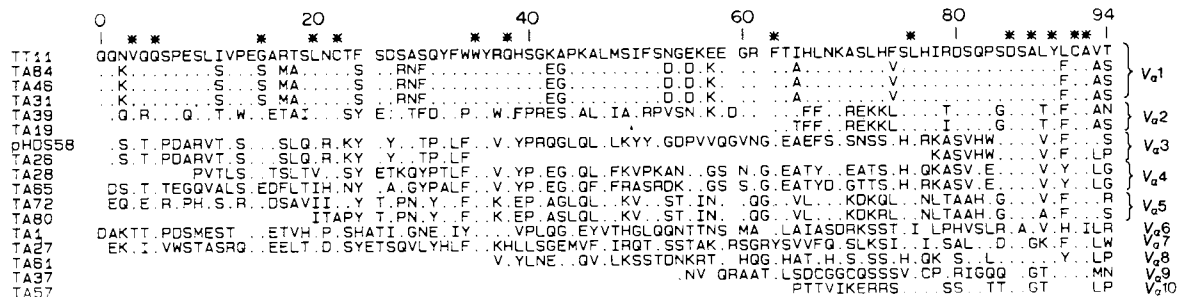
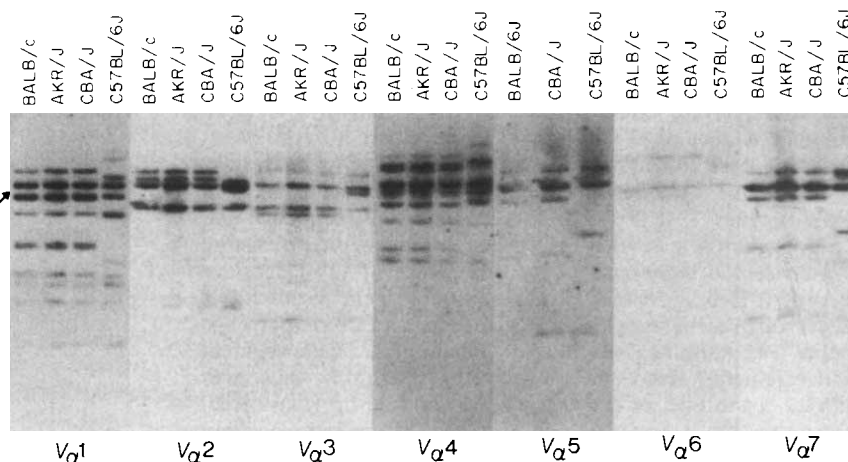


Fig. 2 Protein sequences of 17 V_{α} gene segments. Clone designations (left) and V_{α} subfamily designations (right) are given. Dots indicate that the sequence is identical to that of clone TT11. The blanks indicate sequence gaps introduced to maximize homology. The asterisks indicate residues that are relatively conserved in V_H , V_L , V_B and V_{α} regions.

Fig. 3 Southern blot analysis of mouse germline DNA with complete α -cDNA clones from seven V_{α} subfamilies. Liver DNA from four inbred strains of mice was isolated⁴⁰ and *Bam*HI-digested. Similar results were obtained using liver DNA digested with *Eco*RI and *Hind*III (not shown). The 9.8-kilobase C_{α} -hybridizing bands on each filter have been aligned here and are indicated by an arrow. As the DNA migration distances varied slightly from gel to gel, hybridizing bands at the same location on different filters do not necessarily contain DNA fragments of precisely the same size. Each DNA digest (10 μ g) was electrophoresed on 0.7% horizontal agarose gels and transferred to nitrocellulose⁴¹. Hybridizations were for 24 h at 37 °C in 50% formamide, 0.8 M NaCl, 0.1 M Tris pH 7.5, 5 $\times$ Denhardt's, 100 μ g ml⁻¹ denatured salmon sperm DNA, 10% dextran sulphate and 0.5 μ g ³²P-labelled nick-translated cDNA probe. Filters were washed at 68 °C with 1 $\times$ SSC containing 0.1% SDS.



*Eco*RI fragments as probes (Fig. 3). Because some of the α -cDNAs contain only a small segment of V_{α} sequence (Fig. 1), only seven of the V_{α} subfamilies were analysed. Assuming that each distinct band represents at least one separate V_{α} gene segment, the V_{α} subfamilies analysed here range in size from 1 to 10 members, and the minimum number of bands identified for all seven subfamilies is 40. This distribution is similar to that seen in the immunoglobulin κ - and λ -chain gene families and contrasts with the V_{β} gene family, where six of eight subfamilies analysed contained just one member, and the total V_{β} repertoire is believed to be <21 members^{11,12}.

Three identical V_{α} sequences have been observed to be associated with three distinct J_{α} segments, a surprising result for which we can offer no explanation apart from the possibility that rearrangement and expression of this V_{α} sequence may be preferred in the thymus. The remaining 12 V_{α} sequences are all distinct from one another (Fig. 2).

Restriction length polymorphism is observed between inbred C57BL/6J mice and the three other strains of mice analysed (Fig. 3). Differences are seen with all seven V_{α} probes. In addition, some subfamilies in different mouse strains appear to differ in their V_{α} gene segment numbers; for example, compare the C57BL/6J strain with the others (Fig. 3). The immunoglobulin H- and κ -chain gene families also exhibit extensive restriction length polymorphism, whereas the V_{β} gene family shows very limited polymorphism. Variation in band number within particular subfamilies probably represents the duplication and/or deletion of V_{α} gene segments in the various inbred strains of mice. These data are consistent with the hypothesis that multi-membered V subfamilies may undergo gene expansion and contraction by homologous but unequal crossing-over (see ref. 11).

Numerous J_{α} segments

Of the 17 J_{α} sequences described in this paper and two from the literature^{18,19}, 18 different J_{α} sequences have been identified (Fig. 4). As we found only one repeat, the repertoire of J_{α} gene

segments is probably much larger than 18. We believe that each of these different sequences represents a distinct germline J_{α} gene segment as they differ considerably from one another in sequence. The J_{α} segments exhibit a range of similarity at the protein level (32–72%) comparable with that for J_{β} segments (33–75%) and somewhat lower than that seen for J_{κ} (55–85%), J_H (57–80%) and J_{λ} (70–85%) segments. The J_{α} gene segment repertoire is larger than those of the β -chain (12) or immunoglobulin (4–5) genes. The large repertoire and diversity of the J_{α} gene segments may have important implications for antigen/MHC recognition, as the J segments in immunoglobulins usually fold to form a contact point of the antigen-binding site.

Nonproductive thymic V_{α} transcripts

Nonproductive α -cDNAs may arise by joining events which place the J gene segment in an improper translational reading frame with respect to the V gene segment, by rearrangement of a pseudogene segment or by initiation of transcription 5' to a germline J gene segment or 5' to an incomplete D - J rearrangement. Of the 15 α -cDNAs that have V_{α} gene segments, 5 seem to be derived from nonproductive transcripts. Three of these may have resulted from the rearrangement of a pseudogene. One clone, TA37, has a frameshift within the V_{α} gene segment caused by a single nucleotide deletion at position 317 (Fig. 1). The TA80 clone seems to have a base substitution that has changed the cysteine residue at position 22 to alanine (Fig. 2). This cysteine residue is invariant in immunoglobulins and β -chains and is believed to be important for stabilizing the V domain structure. In addition, the V_{α} and J_{α} gene segments have joined so that the J_{α} gene segment is out of the proper translational reading frame. Thus the TA37 and TA80 clones probably represent transcripts derived from the rearrangement of pseudogenes. A third clone, TA26, has a V_{α} gene segment virtually identical to the previously published pHDS58 V_{α} gene¹⁹ apart from a large deletion in the centre of the V_{α} region encompassing 43 residues and creating a translational frame-

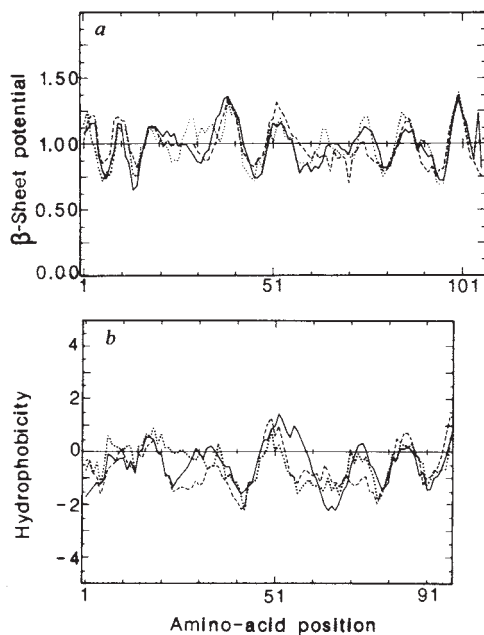


Fig. 6 Secondary structural analyses of V_α (solid lines), V_H (dotted lines) and V_β (dashed lines) regions. *a*, Plot indicating the β -pleated sheet-forming potential of V_β , V_H and V_α segments using the algorithm of Chou and Fasman^{34,35}. *b*, Plot of the relative hydrophobicity of the side chains of the amino acids constituting the V_α , V_β and V_H regions, using the algorithm of Kyte and Doolittle³³. The V_β sequences and V_H sequence compilations are from Barth *et al.*¹¹.

diversity has any functional significance for the T-cell antigen-binding receptor.

Structure of the V_α region

We have analysed the primary and secondary structures of the V_α regions to determine whether they resemble the V regions of β -chains and immunoglobulins.

Both the V_α and V_β regions share many conserved residues with both the V_H and variable light-chain (V_L) regions of immunoglobulins, including 14 residues in the V and 4 residues in the J regions (marked by asterisks in Figs 2, 4). An analysis of the three-dimensional structures of the V domains in several immunoglobulin molecules demonstrates that there are many highly conserved residues important for stabilization of V_L - V_H interactions and for intrachain V_L or V_H interactions^{29,30}. Most of these residues are conserved in the V_α and V_β regions.

Another approach to elucidate primary structural patterns in the T-cell receptor and immunoglobulin V regions is the variability plot of Wu and Kabat³¹; this analysis examines the distribution of variation of each residue position between members of a set of similar sequences. In immunoglobulin and β -chain V regions, two regions of relative hypervariability are noted, with

a third hypervariability³¹ region positioned at the junction of the V, D and J gene segments^{11,32}. A variability plot of the V_α sequences does not differ dramatically from the V_H and V_β variability plots (Fig. 5 and refs 11, 12). With the limited number of V_α sequences analysed, one must be cautious in drawing generalizations for the overall variability in this set is high. However, the regions corresponding to the two classically defined hypervariable regions (residues 30–35 and 51–60) seem to have increased variability. We note two additional areas of hypervariability (residues 8–9 and 66–71) but feel their existence is uncertain until more V_α -sequences are available for analysis. The junction of the V_α and J_α segments generates an additional hypervariable region (not shown).

Two further analyses have been conducted on the V_α sequences to assess the nature of their secondary structure. The Kyte-Doolittle hydrophobicity profile³³ plots the distribution of the relative hydrophobicities of the amino-acid side chains of a particular protein. The Chou-Fasman method^{34,35} determines the relative potential for β -pleated sheet formation. Both these analyses measure properties that are thought to reflect important secondary structural features of the protein. The Kyte-Doolittle hydrophobicity and the Chou-Fasman plots of the V_α , V_β , V_H and V_κ (not shown) sequences are very similar (Fig. 6). The plots represent averages of multiple sequences to minimize unusual individual variations. These data indicate that the secondary structures of the V_α , V_β , V_H and V_κ regions are similar to each other.

Thus, several lines of evidence suggest that general primary and secondary structural features are conserved in the V_α , V_β , V_H and V_κ regions: (1) they share highly conserved amino-acid residues, most of which are important for stabilization of the highly conserved immunoglobulin fold; (2) the variability plots demonstrate variability consistent with three hypervariable regions; and (3) the hydrophobicity and Chou-Fasman plots suggest similar potential for β -pleated sheet formation. These data suggest that the T-cell antigen receptor and immunoglobulin molecules have similar structure. Moreover, as immunoglobulins may also be MHC-restricted³⁶, it is unnecessary to postulate any special sites, apart from the classical antigen-binding site, for binding properties of T-cell MHC-restricted antigen receptors.

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Early *Homo erectus* skeleton from west Lake Turkana, Kenya

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The most complete early hominid skeleton ever found was discovered at Nariokotome III, west Lake Turkana, Kenya, and excavated in situ in sediments dated close to 1.6 Myr. The specimen, KNM-WT 15000, is a male Homo erectus that died at 12 ± 1 years of age, as judged by human standards, but was already 1.68 m tall. Although human-like in many respects, this specimen documents important anatomical differences between H. erectus and modern humans for the first time.

DURING the course of palaeontological exploration on the west side of Lake Turkana, Bw. Kamoya Kimeu found a small fragment of hominid frontal bone exposed on the surface at the site of Nariokotome III, on the south bank of the Nariokotome River. The approximate latitude and longitude of the site are $4^{\circ}08' \text{ N}$, $35^{\circ}54' \text{ E}$ (Fig. 1). Near the site the Plio-Pleistocene beds strike $\text{N } 7^{\circ} \text{ E}$ and dip 5° to the west. Exposures are reasonably good along the south bank of the Nariokotome where a section was measured to establish the stratigraphic position of the hominid (Fig. 2). Several tuffs occur within this section that have been correlated with tuffs elsewhere in the Turkana Basin on the basis of their chemical composition. The hominid derives from a siltstone that immediately overlies a tuff identified as a component ash of the Okote Tuff complex of the Koobi Fora Formation. The age of this tuff is $\sim 1.65 \text{ Myr}^{1,2}$. An ash that correlates with Tuff L of the Shungura Formation (Chari Tuff of the Koobi Fora Formation) dated at $1.39 \text{ Myr}^{3,4}$ lies 34 m above the hominid level. An un-named tuff dated at 1.33 Myr^4 lies 46 m above the specimen. Thus, the hominid is probably very close to 1.6 Myr in age.

The strata consist predominantly of pale yellowish-brown sandstones and siltstones and very pale yellowish-brown to medium-brown siltstones. The sandstones and siltstones are either laminated or massive. The tuff that underlies the hominid fills cracks in an underlying sandy siltstone and contains small-to-medium-scale trough crossbeds truncated at their tops and overlain by siltstone. A small lens of fine tuffaceous sand that lies $\sim 1 \text{ m}$ above the hominid level contains abundant amphioxea and amphistrongyla of freshwater sponges. A tuff that lies 6.7 m above the hominid level contains reworked molluscs at the base and a sandstone 13.8 m above the hominid is capped by an ostracod-rich layer 10 cm thick. Mammalian fossils are rare at this locality, the most abundant vertebrate fossils being parts of small and large fish. The depositional environment was evidently an alluvial plain of low relief, consistent with the fossil fauna in the section. It is likely that the plain was only slightly higher in elevation than a lake which existed nearby, such that with only minor changes in lake level, typical lacustrine forms (for example, ostracods, molluscs) could invade the area.

Twenty-five other vertebrate-bearing sites were located and collected during the 1984 season. Individual sites were labelled by the name of the ephemeral river draining their exposures and were further numbered sequentially in order of their discovery (Table 1). As at Koobi Fora, the Okote Tuff was used as a marker horizon in the subdivision of the fossiliferous succession. Fossil assemblages were retrieved from horizons a short depth below the Okote Tuff at five localities and slightly above it at eight localities (see Table 2). Appreciably fewer taxa are represented at these new localities than at equivalent places at Koobi Fora, but this may be an artefact of sample size. The distribution of identifiable species at sites on both sides of the lake, specifically the presence of *Deinotherium bozasi*, *Elephas recki ileretensis*, *Diceros bicornis*, *Metridiochoerus andrewsi* and

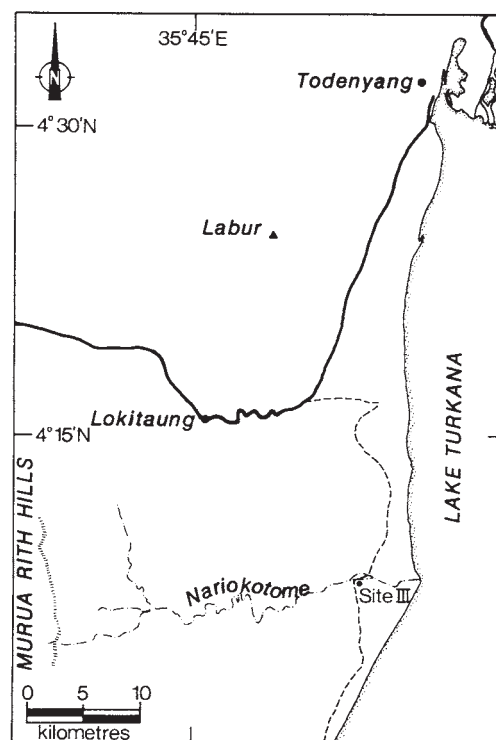


Fig. 1 Map of northern Kenya to show location of site Nariokotome III.

Gazella janenschi only at horizons below the Okote tuff and *Metridiochoerus compactus* only at horizons above this tuff, provides a measure of correlative support for geochemical analyses of the tephra. *Tragelaphus scriptus* is the only species from this part of the section on the west side of the lake that is not represented yet at Koobi Fora.

Following the initial discovery, screening and washing of surface float and pebble lag led to the recovery of most of the hominid calvaria. The facial skeleton was found just eroding out from tuffaceous sediments. Excavation of an area $\sim 5 \times 6 \text{ m}$ has led so far to the recovery of the mandible, several isolated teeth and much of the postcranial skeleton. A list of the parts found so far is given in Table 3. The site plan of the excavation (Fig. 3) shows that the skeleton was dispersed before final sedimentary burial. The bones were found in a layer of tuffaceous silt of variable thickness deposited on a more indurated, flat-lying tuffaceous sand with orange root casts. The top of the fossiliferous horizon shows many signs of bioturbation and several of the bones were found broken or lying in positions suggesting that they had been trampled by large mammals. The

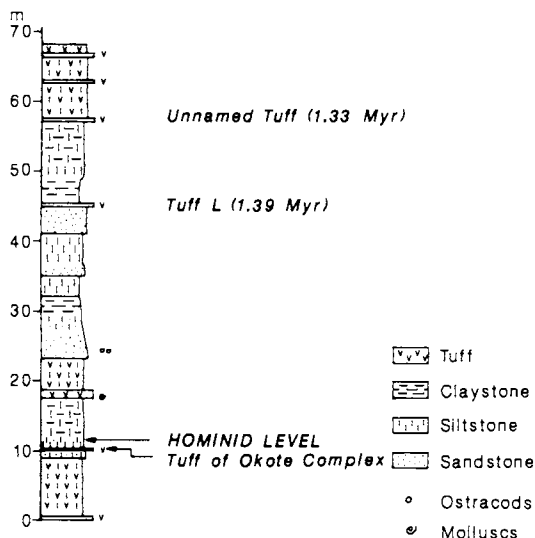


Fig. 2 Section at site Nariokotome III to show stratigraphic position of KNM-WT 15000.

general alignment of the long bones, separation of elements once in close articulation and the linear dispersal of the skeleton over at least 7 m, indicate minor water transport (see Fig. 3). Postmortem damage is seen and some parts of the bones are crushed, particularly where there is much cancellous tissue. There are no signs of carnivore or scavenger damage and the only pathology is a minor amount of alveolar resorption on the right mandibular body which took place before the right dm_2 was shed. The only other fauna found so far in the fossiliferous bed are many opercula of the swamp snail *Pila*, a few bones of the catfish *Synodontis* and two fragments of indeterminate large mammal bone, although equid and pygmy hippopotamus remains were found on the surface here.

The cranium has been assembled from about 70 pieces. The only main pieces missing are the nasals, ethmoid, lacrimals, central parts of the supraorbital tori, parts of the sphenoid and some parts of the vault, the two largest being bits of the right frontal near bregma and right parietal near lambda. The sutures were all unfused. The facial skeleton was positioned on the braincase by using the mandible. The mandible is undistorted, but the calvaria has a slight overall plastic deformation with the upper part displaced to the left. Only a preliminary cranial reconstruction has been possible so far and the capacity has not been measured. Table 4 lists the teeth found so far and their measurements. They are all close to the mean size recorded for *Homo erectus* from Zhoukoudian⁵. The M3 germs will have to be examined radiologically. Several of the teeth were excavated separately and returned to their alveoli. The alveoli for the deciduous upper canines are still visible lateral to the permanent canine alveoli, and the left upper canine was in the process of erupting. The degree of root development of the isolated teeth, the tooth wear stages and the eruption stage are equivalent to that in 12 ± 1 -year-old modern human males⁶. The canines erupted later than the M2s, and although this is not usual, it is a known human condition⁷. The dental age is matched in the postcranial skeleton where all epiphyses are unfused and the incongruity between growth-plate surfaces suggests that much further growth would have been possible. The cranium does not show strong tori, temporal or nuchal lines because it was immature at death. Despite this, its tori are thicker, its palate broader and its facial skeleton bigger and more massive than those of the presumed female cranium KNM-ER 3733 (ref. 8), which is from a roughly equivalent time horizon in the same basin. We take this to mean that there was probably considerable sexual dimorphism in the crania of early *H. erectus*. It is conceivable that this individual, had it lived to maturity, might have developed as strong and robust a cranium as that of Olduvai Hominid 9 (ref. 9).

Table 1 Fossiliferous sites west of Lake Turkana

Locality	Abbreviation	Horizon
Kaitio I	KI I	Indet.
Kaitio II	KI II	Indet.
Kalachoro I	KL I	Below Chari Tuff
Kalachoro II	KL II	Below Chari Tuff
Kalachoro III	KL III	Below Okote Tuff
Kalachoro IV	KL IV	Indet.
Kalachoro V	KL V	Below Chari Tuff
Kalachoro VI	KL VI	Below Okote Tuff
Kalakodo	KK	Below KBS Tuff
Kangaki	KG I	Below KBS Tuff
Kangaki II	KG II	Below KBS Tuff
Loruth Kaado I	LK I	Below Tulu Bor Tuff
Loruth Kaado II	LK II	Below Tulu Bor Tuff
Loruth Kaado III	LK III	Below Chari Tuff
Loruth Kaado IV	LK IV	Below Okote Tuff
Nachakui I	NC I	Above Chari Tuff
Nachakui II	NC II	Above Chari Tuff
Nachakui III	NC III	Indet.
Nanyangakipi	NN	Just below/above Okote Tuff
Nariokotome I	NK I	Galana Boi Beds
Nariokotome II	NK II	Below Chari Tuff
Nariokotome III	NK III	Immediately above Okote Tuff
Nariokotome IV	NK IV	Below Chari Tuff
Natoo	NT	Below Okote Tuff
Nyaena Engol I	NY I	Below Okote Tuff
Nyaena Engol II	NY I	Indet.

Indet., indeterminate horizon.

Previous *H. erectus* postcranial material has been either fragmentary, not definitely associated, disputed as to species or diseased. From Trinil, Indonesia¹⁰, there are several fragmentary and one complete (but pathological) femora. Despite the fact that it was these specimens that led to the species name, there are doubts as to whether they are *H. erectus*¹¹ with the most recent consensus being that they probably are not. Until recently the only *H. erectus* postcranial bones from China were from Zhoukoudian¹² and these were very fragmentary, with no complete lengths or articular surfaces. The reported clavicle is probably not even primate¹³. Recent newspaper accounts report an *H. erectus* cranium, innominate, ulna, three vertebrae, a rib, some hand and foot bones, a patella and a partial innominate from Yingkou, Liaoning Province, China, which are said to be 200,000 yr old^{14,15}.

H. erectus postcrania have also been found at Olduvai Gorge, Tanzania, and East Turkana, Kenya. An associated fragmentary left innominate and femoral shaft (OH 28) from Bed IV Olduvai have been described as belonging to this species¹⁶. Tibial and femoral shafts (OH 34) from Bed III Olduvai¹⁷ may belong, but are so badly eroded that their analytical value is slight. An undescribed ulna (OH 36) from Bed II Olduvai has also been attributed to this species¹³. There are many postcranial bones from East Turkana^{18,19}. Only one is definitely associated with complete enough cranial remains to be absolutely certain of attribution and, unfortunately, that individual had suffered from a disease which affected the postcranial skeleton^{19,20}. The East Turkana specimens are mainly isolated and/or fragmentary.

The finding of KNM-WT 15000 will allow not only the study of the morphology and proportions of this individual, but also allow firmer attributions and enhance the analytical value of very many other isolated or fragmentary specimens. Recognizing that the excavation has yet to be completed and that this will probably result in the recovery of more parts, a complete assessment of this individual will not yet be attempted. There are, however, some points which can confidently be made. The stature of the individual has been estimated by using regression equations developed on modern human adult males²¹ to be 1.68 m (caucasian) or 1.64 m (blacks). This individual would not have been quite that tall, because the cranium does not have the great height of modern *Homo sapiens*. Despite the young age of the individual, the length of the long bones are very close

Table 2 Faunal list

Taxon	West Turkana		East Turkana	
	KLIII, KLIV, LKIV, NY, NN	NT, LKIII, KLI, KLII, KLV, NKIII	Below Okote	Above Okote
<i>Pisces</i>	✓	✓	✓	✓
<i>Crocodylus</i> sp.		✓	✓	✓
<i>Euthecodon brumpti</i>		✓	✓	✓
<i>Trionyx</i> sp.	✓	✓	✓	
<i>Geochelone</i> sp.		✓	✓	✓
<i>Aves</i>	✓	✓	✓	✓
<i>Hystrix</i> sp.		✓	✓	
<i>Theropithecus oswaldi</i>	✓	✓	✓	✓
<i>Homo erectus</i>		✓	✓	✓
<i>Canis</i> sp.	✓		✓	
<i>Carnivora</i> gen. indet.		✓		
<i>Deinotherium bozasi</i>	✓		✓	
<i>Elephas recki ileretensis</i>	✓		✓	
<i>Elephas recki recki</i>		✓		✓
<i>Hipparion ethiopicum</i>		✓	×	×
<i>Equus koobiforensis</i>	✓	✓	✓	
<i>Diceros bicornis</i>	✓		✓	
<i>Ceratotherium simum</i>	✓		✓	✓
<i>Metridiochoerus andrewsi</i>	✓		✓	
<i>Metridiochoerus modestus</i>	✓		✓	✓
<i>Metridiochoerus compactus</i>		✓		✓
<i>Kolpochoerus limnetes</i>		✓	✓	✓
<i>Hexaprotodon karumensis</i>	✓	✓	✓	✓
<i>Hippopotamus gorgops</i>	✓	✓	✓	✓
<i>Hippopotamus aethiopicus</i>	✓	✓	✓	✓
<i>Aepyceros</i> sp.	✓	✓	✓	✓
<i>Megalotragus</i> sp.		✓	✓	✓
<i>Connochaetes</i> sp.	✓	×	✓	✓
<i>Damaliscus</i> sp.	✓	✓	✓	✓
<i>Pelorovis</i> sp.	✓	✓	✓	✓
<i>Pelorovis olduwayensis</i>		×	✓	✓
<i>Syncerus caffer</i>	×	×	×	
<i>Tragelaphus strepsiceros</i>	✓	✓	✓	✓
<i>Tragelaphus scriptus</i>	✓			
<i>Menelikia lyrocera</i>		✓	✓	✓
<i>Kobus sigmoidalis</i>	✓		✓	✓
<i>Kobus kob</i>		✓	✓	✓
<i>Kobus leche</i>		?	✓	✓
<i>Antidorcas recki</i>	✓		✓	✓
<i>Gazella janenschi</i>	✓		✓	✓
<i>Gazella praethomsoni</i>	✓		✓	✓
<i>Gazella</i> sp.		✓		

✓, Species present; ×, comparable species present; ?, stratigraphic position uncertain.

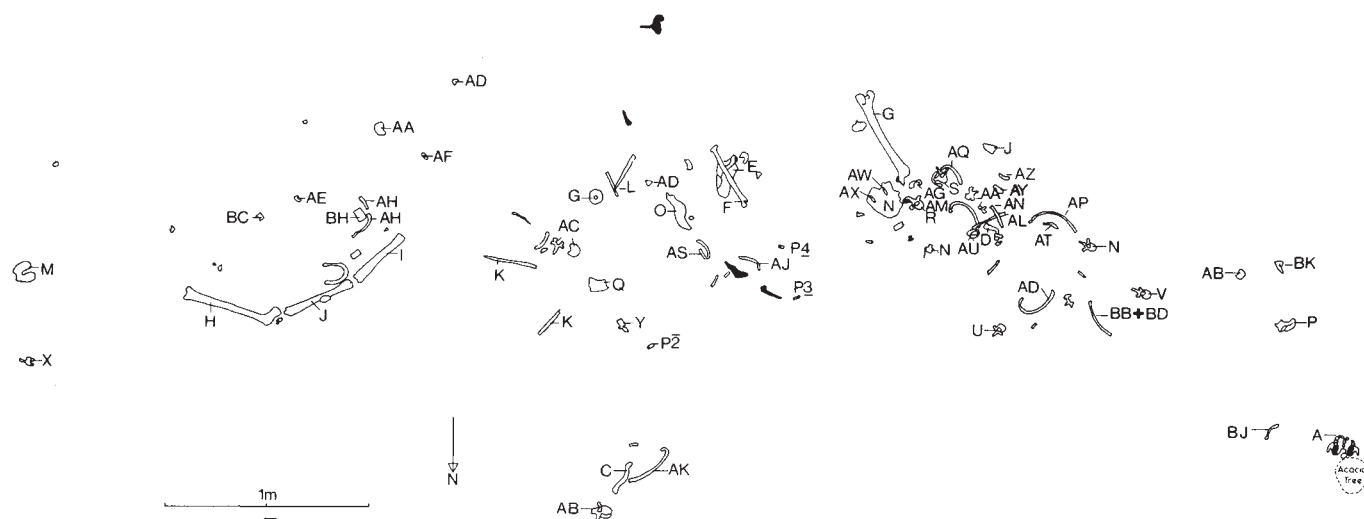


Fig. 3 Detail of site plan showing distribution of *H. erectus* bones. Major hominid parts are as lettered in Table 3. Non-hominid bones, mostly fish, are in solid.

Table 3 Skeletal parts of KNM-WT 15000

Cranium (A)	Right rib 4 (AI, BD, BE)
Mandible (B)	Right rib 5 fragment (BJ)
Cervical vertebra 7 (R)	Right rib 6 (AL)
Thoracic vertebra 1 (S)	Left rib 7 (AP)
Thoracic vertebra 2 (T)	Right rib 7 (AK)
Thoracic vertebra 3 (U)	Left rib 8 (AU)
Thoracic vertebra 6 or 7, spine and laminae (BI)	Right rib 8 (AM)
Thoracic vertebra 8 (V)	Left rib 9 (AO)
Thoracic vertebra 9 (W)	Right rib 9 (AS)
Thoracic vertebra 10 (X)	Right rib 10 (AJ)
Thoracic vertebra 11 (Y)	Left rib 11 (AN)
Lumbar vertebra 1, body and right lamina and inferior facet (AR, BA)	Left clavicle (C)
Lumbar vertebra 2 (AA, AV)	Right clavicle (D)
Lumbar vertebra 3, pedicle laminae and spine (Z)	Left scapula, spine and axillary border (BK, BL)
Lumbar vertebra 4 (AB)	Right scapula (E)
Lumbar vertebra 5 (AC)	Right humerus (F)
Sacral vertebra 1 (AD)	Left ilium (L, BF, BG)
Sacral vertebra 3, laminae and spine (BB)	Left ischium (Q)
Sacral vertebra, right half (BC)	Right ilium (O)
Sacral vertebra 5 (AE)	Right ischium (P)
Coccygeal vertebra 1 (AF)	Right pubic fragments (AW, AX)
Left rib 1 (AG)	Left femur (G)
Right rib 1 (AY, AZ)	Right femur (H, M)
Left rib 2 (AQ)	Left tibia (I)
Right rib 2 (AH)	Right tibia (J)
Left rib 3 (AT)	Left fibula (K, BH)
	Right fibula (L)

to the means for white North American adult males given by Schultz in 1937 (ref. 22). We have examined casts and originals of other early *H. erectus* postcranial bones and find that all of them are large. Stini²³ noted current secular trends of increase in body size in many human populations and wondered whether the trend was revealing a genetic potential left over from early hunter and gatherer ancestors. The new *H. erectus* data support this hypothesis.

The vertebrae show some interesting differences from those of modern humans. The spinous processes on all vertebrae recovered so far are relatively longer and less inclined inferiorly than their modern counterparts and the laminae are much less broad. This means that the marked imbrication of the laminae and spines of adjacent vertebrae (particularly the thoracic series) so typical of *H. sapiens* is much less marked. The vertebral foramen is smaller relative to vertebral body size in the lower cervical and thoracic series, unlike that of modern humans, but about the same in the lumbar series. The inferior articular facets of the fifth lumbar vertebra face almost directly anteriorly and are slightly concave, in contrast to the usual human condition in which the surfaces face laterally and are cylindrically convex²⁴.

Although we have yet to recover sufficient parts of the pubic bones to complete a full reconstruction, the innominates are very similar indeed to OH 28 and KNM-ER 3228 (refs 16, 25), but the strong iliac pillar had not yet developed. The sacrum is represented by S1 and S5, the spine of S3 and half of S4. Although we can reasonably estimate the sacral width, precise alignment with the pelvis must wait for the recovery of S2. There was a remarkable degree of iliac flare²⁶; this flare is concordant with the extremely long femoral necks, which are relatively as long as those of robust australopithecines²⁶. The biomechanical advantage of the abductor mechanism²⁶ was much enhanced relative to the condition in *H. sapiens*. The biomechanical neck length of 85 mm is well over 3 s.d. from the mean of a sample of *H. sapiens*²⁶. As well as having a long femoral neck, the neck-shaft angle is very small at 110°, being 5 s.d.s from the mean of the same *H. sapiens* population. The femoral head diameter at 44.0 mm is almost exactly on an *H. sapiens* mean²⁷ and scales with femoral length in the same way as *H. sapiens* and *Australopithecus boisei*²⁸.

Martin²⁹, following a stimulating discussion of human and great ape brain growth, suggested that early *H. erectus* could

have produced their adult brain sizes by modification of the rate and/or extent of fetal growth without any need for significant postnatal postponement of fetal growth patterns as found in humans. This is dependent, however, on *H. erectus* pelves having allowed the birth of an infant as large as those of *H. sapiens*. Modern human populations show little, if any, sexual dimorphism in the absolute width of the sacrum³⁰, a major determinant of pelvic inlet size. The interacetabular distance in WT 15000 is very small and surely correlated both with the great iliac flare and long femoral necks. It seems likely to us that early *H. erectus* birth-canal diameters would have been significantly smaller than in *H. sapiens* and that passage of a modern-sized, full-term fetus would have been impossible. The continuation after birth of fetal growth rates, found only in

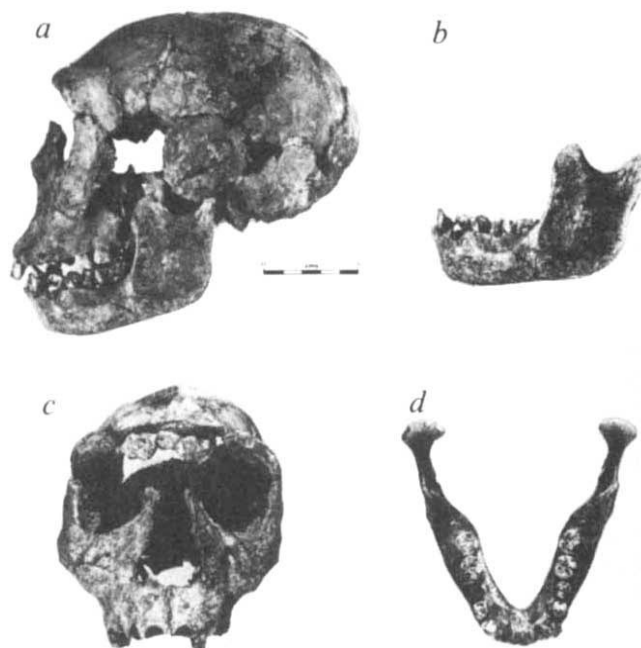


Fig. 4 KNM-WT 15000. a, Left lateral view of skull; b, left lateral view of mandible; c, frontal view of cranium; and d, occlusal view of mandible.

Table 4 Tooth measurements (mm)

	Right		Left	
	Mesiodistal	Buccolingual	Mesiodistal	Buccolingual
Mandible				
C	8.8	9.0	8.6	9.4
P ₃	9.0	10.1	8.5	10.1
P ₄	—	—	9.0	9.5
M ₁	11.9	11.1	12.2	10.9
M ₂	12.4	11.4	12.2	11.5
Maxilla				
I ²	7.9	8.5	7.9	8.3
P ³	8.3	11.5	—	—
P ⁴	8.2	11.5	—	—
M ¹	12.2	11.8	11.1	11.8
M ²	12.5	12.6	13.3	11.7

humans, must have been part of the developmental mechanism of *H. erectus*. Already then, by 1.6 Myr ago, the secondarily altricial condition (which leads to increased infant dependency) must have been present.

This skeleton has already shown that our views of early

H. erectus morphology must be modified. With the possibility of finding more parts when the excavation is continued and with further analysis, KNM-WT 15000 will provide a firmer foundation for the identification and understanding of unassociated postcranial bones and early hominid growth patterns. Except for those representing the latest stages of our evolution, it is also the first fossil hominoid, let alone hominid, in which brain and body size can be measured accurately on the same individual. These two crucial variables, on which so much speculation about human origins and behaviour has been based, can now be determined for at least one individual early hominid.

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LETTERS TO NATURE

Age of the Okote Tuff Complex at Koobi Fora, Kenya

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The Okote Tuff Complex crops out in the Koobi Fora region next to the north-eastern shores of Lake Turkana, northern Kenya. It occurs in the upper part of the ~450 m thick, essentially flat lying, Plio-Pleistocene sequence of fluvial, deltaic and lacustrine sediments comprising the Koobi Fora Formation¹⁻³. Many important archaeological sites and hominid fossils, including the *Homo erectus* finds discussed in the accompanying papers^{4,5}, are stratigraphically close to the level of the Okote Tuff Complex⁶ or its correlatives, so that its precise numerical age is of great interest. Previously published isotopic ages, mainly by the ⁴⁰Ar/³⁹Ar dating technique, exhibited a very wide scatter⁷. Here we report comprehensive K-Ar data showing that pumices within one of the tuffs of the Okote Tuff Complex have an age of 1.64 ± 0.03 Myr, which is in the earliest Pleistocene⁸.

The type locality for the Okote Tuff Complex is within area 131, adjacent to Karari Ridge, 20-km east of Lake Turkana (Fig. 1). The unit was formally named by Findlater² and discussed further by Cerling and Brown³. A stratigraphical section measured in this area is shown in Fig. 2, after ref. 5. The Okote Tuff Complex lies about mid-way between the KBS and Chari Tuffs which, in area 131, are separated by ~40 m of section consisting mainly of river channel sands together with some flood plain silts and clays. The Complex has yet to be defined and described in detail. In the field it consists of several sandy

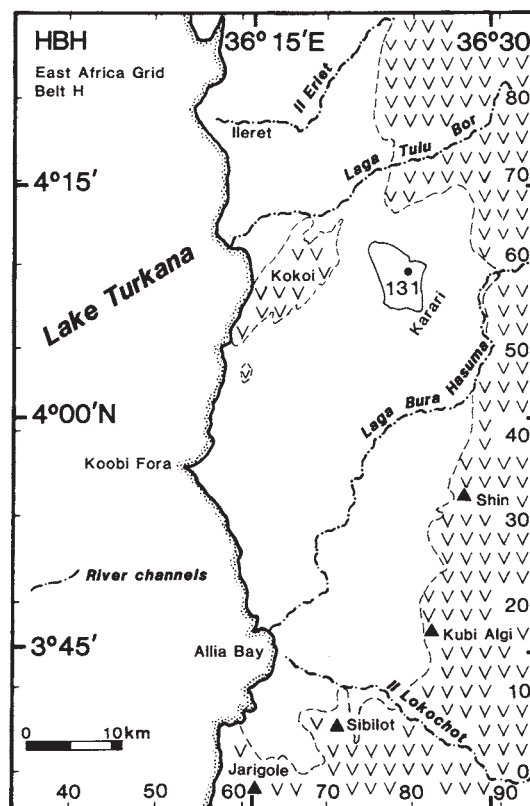


Fig. 1 Outcrop of the Koobi Fora Formation adjacent to the shores of Lake Turkana is indicated by blank area. Type locality for the Okote Tuff Complex is shown by filled circle within fossil collection area 131. Local basement, dominantly Neogene volcanics, is indicated by pattern.

Table 1 K-Ar age data for alkali feldspar phenocrysts from pumice clasts within a tuff bed of the Okote Tuff Complex, area 131, Koobi Fora, northern Kenya

Sample no.	K (wt%)	Radiogenic ^{40}Ar (10^{-11} mol g $^{-1}$)	100 Radiogenic ^{40}Ar Total ^{40}Ar	Calculated age $\pm$ 1 s.d. (Myr)	Average age $\pm$ 1 s.d. (Myr)
81-133	4.757, 4.737	1.395	43.3	1.694 $\pm$ 0.020	1.65 $\pm$ 0.05
		1.320	38.3	1.603 $\pm$ 0.020	
		1.361	33.2	1.652 $\pm$ 0.022	
81-134	4.745, 4.741	1.600	82.6	1.941 $\pm$ 0.018	—
83-265	4.728, 4.744	1.347	54.7	1.639 $\pm$ 0.018	1.65 $\pm$ 0.02
		1.368	47.3	1.665 $\pm$ 0.020	
83-272	4.698, 4.704	1.325	38.8	1.625 $\pm$ 0.020	1.64 $\pm$ 0.02
		1.351	38.6	1.657 $\pm$ 0.020	
83-326	4.712, 4.736	1.357	57.9	1.656 $\pm$ 0.020	1.64 $\pm$ 0.02
		1.338	47.7	1.632 $\pm$ 0.020	
83-328	4.715, 4.723	1.307	31.6	1.596 $\pm$ 0.021	1.62 $\pm$ 0.03
		1.338	25.6	1.634 $\pm$ 0.026	
83-329	4.682, 4.710	1.299	23.9	1.594 $\pm$ 0.025	1.60 $\pm$ 0.02
		1.313	42.4	1.611 $\pm$ 0.019	
83-330	4.694, 4.695	2.011	42.5	2.468 $\pm$ 0.028	—
83-331	4.751, 4.726	1.320	47.9	1.605 $\pm$ 0.019	1.64 $\pm$ 0.04
		1.391	36.9	1.692 $\pm$ 0.023	
		1.345	55.2	1.636 $\pm$ 0.018	
83-332	4.699, 4.702	1.390	60.7	1.704 $\pm$ 0.019	1.70 $\pm$ 0.02
		1.386	63.2	1.699 $\pm$ 0.019	

$\lambda_e + \lambda'_e = 0.581 \times 10^{-10} \text{ yr}^{-1}$, $\lambda_\beta = 4.962 \times 10^{-10} \text{ yr}^{-1}$, $^{40}\text{K}/\text{K} = 1.167 \times 10^{-4} \text{ mol mol}^{-1}$. Mean age (excluding 81-134, 83-330) = 1.64 ± 0.03 Myr. Mass of sample used in Ar extraction in range 0.8 to 1.5 g. Locality: at coordinates 049-124 on 9×9-inch photographic print number 1750 taken in December 1970 by Hunting Surveys, London, where coordinates are millimetres from left edge and from the top edge of the print, respectively. Trimming of the pumices was done in the field to remove the outer centimetre or two of each clast to minimize the possibility of contamination of the samples by detrital feldspar. Some of the pumices were partly calcified. Separation of the feldspars from the pumice clasts was done, after crushing, on the >0.5 mm size fraction by water panning, followed by purification using heavy liquid and magnetic techniques. Most concentrates were washed ultrasonically for 3 min in 7% HF to help remove traces of glass adhering to the feldspar. Purity of >99% was attained. The crystals were quite unaltered and limpid, and exhibit very fine polysynthetic twinning. K and Ar were measured by flame photometry and isotope dilution, respectively. Ar extractions were made in a vacuum system by radiofrequency heating of the sample, contained in a Mo crucible, to temperatures >1,600 °C for 1 h to try to ensure full Ar recovery from the melt. After purification of the Ar, isotope analysis was carried out in a VG-Isotopes MM1200 gas-source mass spectrometer.

rhyolitic tuff beds extending over ~16 m of section. Individual beds are generally 0.7–1.5 m thick. The tuffs were deposited from water in much the same manner as the enclosing detrital sediments.

In a small hill ~100–150 m north of archaeological site FxJj20 at East African Grid HBH793592, at the type locality of the Okote Tuff Complex, rounded pumice clasts of up to ~20 cm in diameter are found in the upper part of a tuff bed 1.6-m thick, designated the Lower Okote Tuff⁵. A relatively large number of pumices was collected from this locality, as each contained some alkali feldspar phenocrysts, ideal for isotope dating by the conventional K-Ar method. Dating techniques used were similar to those described previously⁹.

Results on the alkali feldspar (anorthoclase) concentrates from 10 different pumice clasts are given in Table 1. Duplicate K analyses agree to better than 0.5% in most cases. Replicate Ar measurements show a spread averaging about 2%, with differences of >5% in two cases. For these two samples (81-133 and 83-331), the relatively large spread in their measured Ar contents may be because of minor but variable contamination by detrital feldspar, or because of incomplete extraction of the radiogenic Ar from some of the melts; with the available data it does not seem possible to distinguish between the alternative explanations.

The K contents of the 10 anorthoclase concentrates show a very limited spread, with a mean of 4.72 (+0.02)%; this is good evidence that the pumice clasts were derived from a single volcanic eruption.

Eight of the alkali feldspars yielded K-Ar ages within the range 1.60–1.70 Myr, from which a mean age of 1.64 ± 0.03 Myr is calculated. The remaining two feldspars gave substantially

older apparent ages of 1.94 ± 0.02 and 2.47 ± 0.03 Myr (Table 1). These two results are clearly anomalous, probably to be explained by minor contamination of the mineral separates from old detrital feldspar incorporated within the pumice clasts.

The good agreement of the K-Ar results on eight of the feldspars, yielding a mean age of 1.64 ± 0.03 Myr, is accepted as strong evidence that this records the time of cooling of the feldspars immediately following the explosive volcanic eruption that produced the pumice clasts. Findlater¹⁰ argued that the time between explosive volcanism and deposition of the products is likely to be short in comparison with the time resolution obtainable by the K-Ar dating method. Brown and Feibel⁵ have shown, however, that the glass of the pumice clasts differs significantly in composition from that of the enclosing Lower Okote Tuff, implying that they are products of different eruptions. No tuffs in area 131 have been found that have glass of the same composition as that of the pumices, although a good match is obtained with tuff J4 in the Shungura Formation. As pumice clasts are likely to have been transported by floating¹¹, reworking and final incorporation into younger sediments is a reasonable explanation for their present location. Thus 1.64 ± 0.03 Myr must be the maximum age for deposition of the enclosing Lower Okote Tuff.

This limiting age for a tuff in the lower part of the Okote Tuff Complex is intermediate between that obtained for the Chari Tuff (1.39 ± 0.02 Myr; ref. 12) higher up the sequence, and the age of 1.88 ± 0.02 Myr (refs 9, 12, 13) found for the KBS Tuff ~16 m below, consistent with the stratigraphical position of the Okote Tuff Complex in the sequence.

The KBS Tuff and immediately adjacent sediments are normally magnetized, whereas the overlying sediments, including

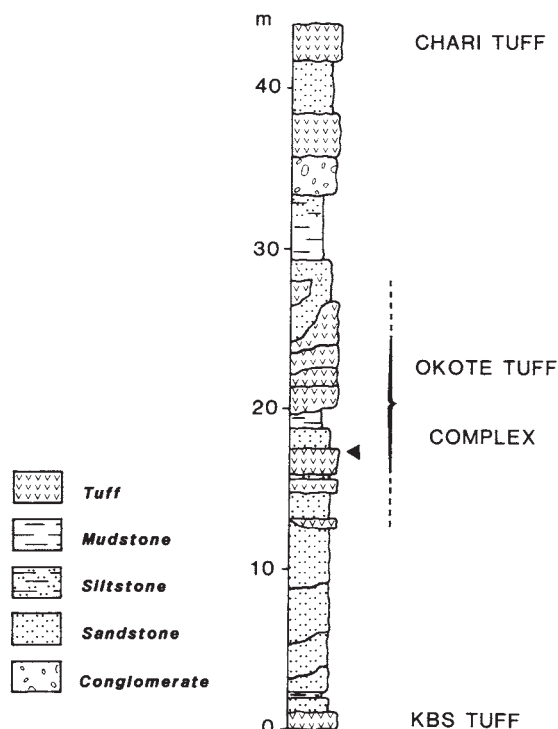


Fig. 2 Stratigraphical section through part of the Koobi Fora Formation in area 131, adjacent to Karari Ridge, after ref. 5. Pumice clasts dated in this study were collected from the Lower Okote Tuff, indicated by the symbol on the section ~17 m above the base.

the Okote Tuff Complex, are of reverse polarity^{14,15}. The palaeomagnetic and age data, therefore, are consistent with deposition of the sequence during the Matuyama reverse chron, the sediments at the KBS Tuff level having been laid down during the Olduvai normal subchron¹⁶.

In the Ileret area and adjacent to Koobi Fora spit (Fig. 1), tuffaceous beds also occur above the KBS Tuff in a stratigraphical position similar to that of the Okote Tuff Complex. Various called Ileret, Lower/Middle and Koobi Fora Tuff Complexes, these are thought to be stratigraphical correlatives of the Okote Tuff Complex^{2,3,5,11}. Cerling and Brown showed³, however, that glass separates from pumice and from tuff display considerable compositional variability, such that definite correlations between individual tuffs in the different areas was not possible. As the compositional variations observed lie on well defined trends, they suggested³ that the tuffs are all genetically related, and, therefore, probably correlatives on a broad time-scale. Studies by Brown and Feibel⁵ have subsequently yielded some correlations. The age derived here for pumices in one of the tuffs in the Okote Tuffs Complex can thus be regarded as providing an approximate guide to the age of the other tuff sequences referred to above.

The well-constrained maximum age for a tuff within the Okote Tuff Complex provides excellent time control for the abundant vertebrate faunas, including hominids, that occur in the upper part of the Koobi Fora Formation. Correlations indicate that the recent important *Homo erectus* find at Nariokotome on the west side of Lake Turkana also was from a stratigraphical level equivalent to that of the Okote Tuff Complex^{4,5}. Together with the dating results reported by McDougall¹² on feldspars from pumice clasts in other tuffs in the sequence, a detailed chronology for the Koobi Fora region is emerging. The successful geochemical correlation of tuffs between the sequences at Koobi Fora, the Lower Omo Valley just north of Lake Turkana (Shungura Formation) and the west side of Lake Turkana, strongly supports the idea that the sediments belong to a single major depositional system within the Turkana Basin^{3,5,17}. The chronology also can be applied throughout the region, with measurements on samples from different parts of the Turkana Basin contributing to the overall picture. The available data

suggest that accumulation of sediments in the Turkana Basin began ~4.1 Myr ago in the early Pliocene, and has continued until the present day, although quite lengthy hiatuses, representing periods up to or exceeding 0.5 Myr, are recognized in individual sequences^{12,17}.

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Stratigraphical notes on the Okote Tuff Complex at Koobi Fora, Kenya

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The Okote Tuff Complex of the Koobi Fora Formation in northern Kenya contains many archaeological sites¹ and many hominid specimens derive from levels nearby². The complex lies between the KBS Tuff and the Chari Tuff, both of which have been precisely dated^{3,4}, and recently McDougall (in the accompanying manuscript⁵) has measured the age of pumice clasts derived from the tuff in which they were deposited. The age is still useful, because a tuff of known stratigraphical position in the Shungura Formation is of the same composition. Here we discuss stratigraphical relations between the Okote Tuff Complex and other related strata at Koobi Fora, in the lower Omo Valley, and west of Lake Turkana based on compositional study of ash layers. This allows us to estimate an age of 1.60 ± 0.05 Myr for the *Homo erectus* skeleton recovered at Nariokotome in 1984 (see accompanying article⁶).

Several conditions have contributed to the difficulty of framing a general stratigraphy in this part of the section, or even to defining the sequence of tuffs which occur. The first is that in many localities only a single tuff or a pair of tuffs is exposed, and it is often difficult to determine the order of tuffs where exposures are poor because many of them occur discontinuously as channel fillings. Many of the tuffs in this interval have reasonably similar composition with respect to both major and trace elements; each of them is variable in composition so that where few analyses are available, samples which actually belong to a single group may be placed into different categories. Some of the tuffs are composed of shards of more than one composition⁷, and fractionation may have occurred either naturally (by differential weathering) or in the laboratory (by bias towards more or less magnetic fractions during electromagnetic separation). Finally,

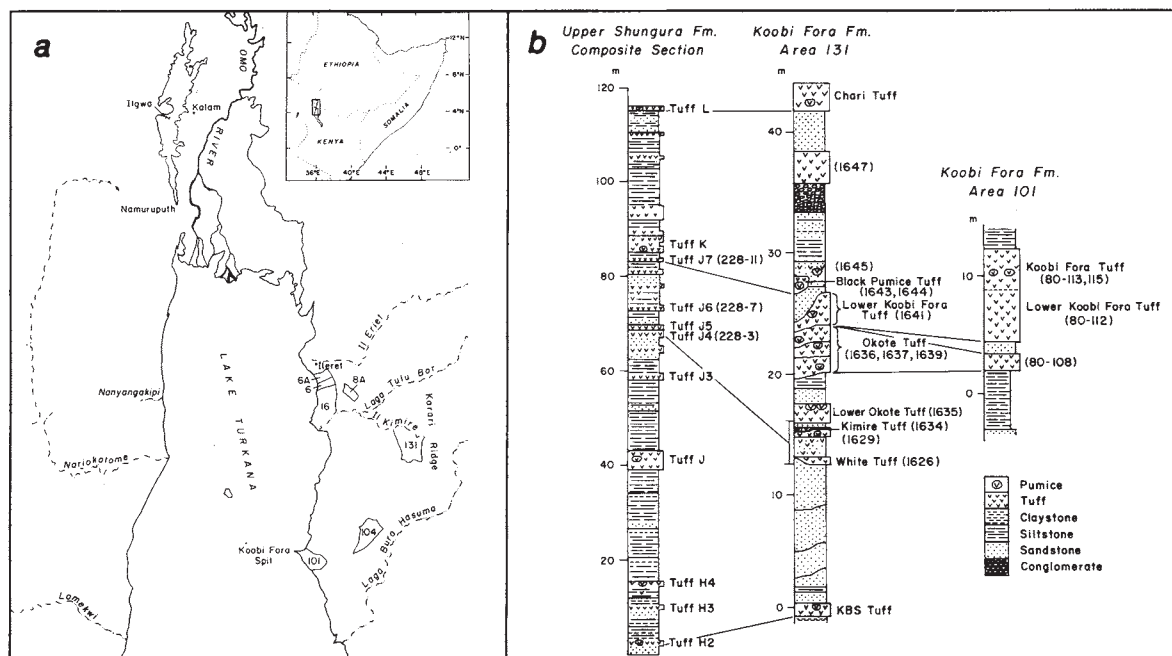


Fig. 1 a, Location map; b, Correlated stratigraphical sections through the Okote Tuff Complex in the lower Omo Valley and the Koobi Fora region.

the sequence is obscured if analyses of pumice and fine-grained ash are considered together; only after separation of the pumice analysis from those of the tuffs does the situation become clearer. These problems can be overcome provided there are enough analyses to determine the variation in composition of a particular tuff, and provided the analyses are done on samples from more than one section so that sequences can be shown to be homotaxial. Stratigraphical statements made here are based on 143 individual analyses of glass from tuffs and from pumices contained within them which lie between the KBS and Chari Tuffs.

There are two key sections on which the present stratigraphical conclusions are based, one situated in area 131 on the Karari Ridge, the other situated in the lower Omo Valley (Fig. 1a, b). In a detailed section in area 131, there are 14 tuffs or tuffaceous layers beginning with the KBS Tuff and ending with the Chari Tuff. We view three layers near the middle of the section as products of a single eruption because they are compositionally identical. The section of the Shungura Formation is composite, but tuffs discussed here were collected from a single section at Ilgwa (see ref. 8); analyses are given in Table 1. A third section located near Koobi Fora spit is necessary to place the Koobi Fora Tuff (defined below) in this sequence.

On the basis of the stratigraphical order of tuffs in these sections, and the composition of the ash fractions, the correlations shown in Fig. 1b have been made. Appended names are explained below. We realize that this stratigraphy is incomplete—indeed we have analysed 10 other tuffs known to lie within this same interval, but which are less well constrained stratigraphically, and thus not reported here.

In previous reports concerned with the stratigraphy of this interval⁹⁻²⁰, the tuffaceous part of the section along the Karari Ridge was referred to as the Okote Tuff Complex (or as the Okote Tuff); at Ileret, the Lower/Middle Tuff Complex (or Middle Ileret Tuff); and near Koobi Fora spit, the Koobi Fora Tuff Complex. With the exception of the Okote Tuff Complex defined by Findlater¹³, these terms are informal. To clarify stratigraphical relations between the three areas at Koobi Fora we here name several tuffs which have formerly been treated as parts of one or another of the complexes. For each newly named tuff we designate a type locality and give a short description; representative analyses of each are given in Table 1.

A tuff in area 104 has been informally known as the White Tuff; it is also present in area 131 where it is the lowest recognized ash layer mapped as the Okote Tuff Complex. We define this tuff as the White Tuff, and designate its type locality as (1750/043, 103) (ref. 21) in area 131 of the Koobi Fora region where the tuff is reasonably well exposed, and where its relation to other tuffs in the Okote Tuff Complex can be seen. This tuff is ~40-cm thick, fine grained, and very pale grey to white. It is composed dominantly of colourless shards with tubular vesicles. Its very pale colour is diagnostic in the field and its composition is unique.

About 2 m higher in the section in area 131 there is a medium-grey tuff ~80-cm thick (K83-1629, Table 1) which we do not name. Filling a channel in the upper part of this tuff is another ash ~40-cm thick which we designate the type locality of the Kimire Tuff, named for the stream in which it is exposed. Photo coordinates of this locality are 1750/054, 131. (Photo coordinates refer to aerial photography HSL KEN 70 6 flown by Hunting Surveys Ltd. The notation is read as photo number/x, -y with the x distance in millimetres from the left hand margin of the image and the y distance in millimetres from the top of the image. A set of these photos exists in the Koobi Fora archives at the National Museums of Kenya in Nairobi.) Shards with spherical bubbles make up about half of the Kimire Tuff; the remainder are striated, bubble junction or bubble wall shards. This tuff cannot be distinguished chemically from the Lower Koobi Fora Tuff; it is separated here because the stratigraphical relations are clear. Isolated samples from other localities could be referred to either the Kimire Tuff or to the Lower Koobi Fora Tuff.

A thin sandstone lies between the Kimire Tuff and the next tuff which we designate the Lower Okote Tuff (Type locality 1750/057, 133). The pumices dated by McDougall⁵ derive from this horizon, but are chemically very distinct from the enclosing ash. The Lower Okote Tuff (1.6 m) is medium grey, fine to medium grained, and composed dominantly of shards with elongate vesicles with a smaller fraction of bubble junction shards. Although similar in composition to some other ashes in this same stratigraphical interval, the Lower Okote Tuff is reasonably well defined compositionally.

There are four pumiceous tuff beds 2 m above the Lower Okote Tuff, which comprise ~6 m of the section. The lower

Table 1 Representative analyses of tuffs and pumice from the Okote Tuff Complex

Sample no.	Location	Weight per cent		Elemental content (p.p.m.)								
		Fe ₂ O ₃	CaO	Ba	Mn	Nb	Rb	Sr	Ti	Y	Zn	Zr
Unnamed Tuff												
83-1647	131	4.89	0.55	143	2,154	148	113	4	2,642	84	191	993
Unnamed Tuff												
83-1645	131	4.89	0.29	48	1,510	139	116	3	2,319	102	211	1,086
Black Pumice Tuff												
81-615	Shin	2.38	1.12	719	1,077	76	83	129	2,692	63	97	708
228-11	Omo	2.46	1.12	740	989	80	77	151	2,731	60	95	661
83-1576	8A	2.91	1.51	751	960	70	77	187	3,792	53	96	701
83-1643	131	2.67	1.07	764	1,024	86	81	108	2,473	63	102	726
83-1644D	131	1.91	0.73	784	792	77	91	80	2,270	61	80	570
Koobi Fora Tuff												
228-7	Omo	5.64	0.27	40	2,112	241	143	3	2,682	127	290	1,576
80-113	101	5.35	0.30	10	1,862	252	120	6	2,176	130	306	1,658
80-115	101	5.39	0.19	<5	1,899	256	127	1	1,969	131	282	1,656
Lower Koobi Fora Tuff												
83-1641	131	4.83	0.30	35	1,463	145	122	11	2,208	106	224	1,164
80-112	101	5.27	0.49	35	1,600	163	107	20	2,413	104	226	1,198
80-163	103	5.00	0.37	35	1,485	142	108	12	2,064	99	215	1,096
Okote Tuff												
83-1636	131	4.75	0.18	10	1,759	242	145	1	1,902	121	265	1,542
83-1639	131	4.76	0.21	21	1,736	228	148	1	1,948	120	265	1,513
83-1637	131	4.69	0.23	12	1,546	214	149	6	1,835	121	255	1,516
80-108	101	4.70	0.46	8	1,610	214	118	4	2,494	114	241	1,396
Lower Okote Tuff												
83-1635	131	4.66	0.14	25	1,105	164	168	2	1,193	127	259	1,540
83-1575	8A	4.80	0.14	<5	1,073	167	157	1	1,097	128	257	1,580
Tuff J4												
228-3	Omo	3.31	0.43	262	816	141	84	4	1,321	102	196	1,176
ANU83-331	131	3.19	0.53	645	906	146	86	10	1,725	108	195	1,134
ANU83-328	131	2.73	0.34	193	721	153	95	3	1,462	111	207	1,022
ANU83-326	131	2.80	0.52	226	705	155	98	7	1,514	115	205	1,035
83-511	Turkana	2.96	0.37	218	710	157	93	1	1,111	116	245	1,047
Kimire Tuff												
83-1634	131	5.00	0.29	45	1,541	136	110	1	1,962	102	216	1,100
Unnamed Tuff												
83-1629	131	4.74	0.55	691	1,751	104	95	5	2,220	76	178	846
83-1629NM	131	4.74	0.52	670	1,635	104	95	4	2,201	77	193	850
White Tuff												
83-1626	131	1.84	0.17	26	247	112	159	2	502	87	117	474
80-169	104	1.86	0.18	<5	242	116	175	1	498	93	120	526
Tuff at Nariokotome below hominid KNM-WT-15000												
84-2957	Turkana	5.18	0.34	97	1,440	135	110	1	2,098	101	221	1,095

three of these are compositionally identical (Table 1), and as these comprise the greatest thickness of ash at this locality we designate these three beds the Okote Tuff. At the type locality (1750/061, 137) the Okote Tuff (4 m) is medium grey and consists of a mixture of shard types. Compositionally this tuff is well defined, and corresponds to a 1.4-m ash layer which lies ~1 m below the Lower Koobi Fora Tuff in area 101.

The fourth Tuff in the sequence described above correlates with the base of the thick tuff in area 101 which has been informally regarded as the type locality of the Koobi Fora Tuff⁸. Here we designate the tuffaceous section in area 101 as the type locality of both the Lower Koobi Fora Tuff and the Koobi Fora Tuff (1343/102, 173). There is no clear stratigraphical break between the lower and the upper part of the thick tuff in area 101, but the base is compositionally distinct from the upper part (see Table 1). In other parts of the Koobi Fora region, ashes of each composition are found as discrete layers. The lowest 4.1 m of section at the type locality is taken as the type of the Lower Koobi Fora Tuff; the upper 3.3 m of section at the same locality is taken as the type of the Koobi Fora Tuff. Both of these tuffs are composed dominantly of shards with tubular vesicles with a few blocky or bubble junction shards. The Koobi Fora Tuff

probably correlates with Tuff J-6 of the Shungura Formation, although there are minor differences in composition (Table 1). In area 131, the Koobi Fora Tuff has not been identified, probably because the Lower Koobi Fora Tuff is terminated there by an erosional surface with at least 3-m relief.

Encased within the sandy section overlying the Lower Koobi Fora Tuff in area 131 is a distinctive tuff composed of both grey and brown shards. There it occurs in a channel lined with ash, filled with black pumice clasts up to 25 cm in diameter, but also contains grey pumice clasts of similar size. We here designate this tuff as the Black Pumice Tuff. It is 0–1 m thick at its type locality (1750/060–139), pale brownish yellow in hand specimen, and composed dominantly of flat bubble junction shards. Compositionally this tuff, though variable, is unmistakable (Table 1); it correlates with Tuff J-7 of the Shungura Formation. (Two other separates have been analysed from the section in area 131 between the Black Pumice Tuff and Chari Tuff (K83–1645; K83–1647; Table 1, Fig. 2), but as they are known only from this locality, we do not name them here.)

Turning to the composition of the pumice clasts, we find that most can be accommodated in the analytical groups defined using ash samples only. One fact becomes immediately clear

from the analyses of pumice clasts—pumices are not confined to the tuff which they match compositionally, but may be found also in tuffs and other sediments at higher stratigraphical levels. For example, the Koobi Fora Tuff contains not only pumice corresponding to its own composition, but also pumices which compositionally match the Lower Koobi Fora Tuff and the Okote Tuff. This causes the problem of interpreting the ages on the pumices from the Lower Okote Tuff, because they do not correspond to the composition of that tuff. Clearly, the measured ages refer to the time of eruption of the pumice, and must antedate the time of deposition of tuff in which they are included. In a general sense, the effect of upward reworking of pumice clasts in a section is to make the entire section appear older than it actually is. In contrast to pumices, which maintain their integrity even during and after reworking, ash layers are not often redeposited as discrete layers. (There are, however, examples of redeposited ash layers in the region.) Hence, we argue that the age on pumice clasts must be applied stratigraphically to the first appearance of an ash in the section which corresponds to the composition of the dated pumice.

The pumices from the Lower Okote Tuff correspond in composition to Tuff J-4 in the Shungura Formation of southern Ethiopia, and also to a tuff exposed west of Lake Turkana, but do not correspond to that of any tuff yet analysed from the Koobi Fora region. We believe that the age reported by McDougall¹ may be applied to Tuff J-4 of the Shungura Formation. Using this age, we can estimate ages of tuffs lying between J-4 and Tuff L in the Shungura Formation by scaling on the basis of stratigraphical thickness. This in turn allows us to set limits on the age of the Lower Okote Tuff, knowing that it is $\leq 1.64 \pm 0.03$ Myr. Using various sections for scaling, the Black Pumice Tuff (Tuff J-7) is found to have an age between 1.52 and 1.57 Myr, hence the Lower Okote Tuff must lie between 1.64 and 1.52 Myr. If a further correlation is accepted (that between Tuff J-6 and the Koobi Fora Tuff), then the age of the Lower Okote Tuff is further constrained to lie between 1.60 and 1.64 Myr, which is virtually the same as the error in the reported age. In this case, the tuff in question is only slightly younger than the pumices included within it even though they differ in composition. The lowest tuff known to have been mapped within the Okote Complex is the White Tuff which can be assigned an age of ~ 1.7 Myr by stratigraphical scaling of the section in area 131.

Detailed correlations between the Karari Ridge and the Ileret area are still not completely clear. The Lower Koobi Fora Tuff and the Koobi Fora Tuff have been identified in areas 6, 6A and 8A in the southern part of the Ileret Area, and these lie above the Lower Ileret Tuff²⁰. We can set no useful lower boundary on the level of the Lower Ileret Tuff, although it is known to overlie the KBS Tuff. The Black Pumice Tuff has also been identified in area 8A. The prominent tuff exposed below the Chari Tuff along the northern part of the Ileret Ridge does not correlate with any of the tuffs reported here, nor with any other known tuff from the region.

In conclusion, the Okote Tuff Complex and the Koobi Fora Tuff Complex as a whole probably lie within the interval between 1.5 and 1.7 Myr. The stratigraphical interval between the KBS Tuff and the Okote Tuff Complex represents a period of no more than 200,000 yr. Several archaeological sites can now be placed into an ordered sequence along the Karari Ridge, and with further work others will undoubtedly be placed as well. The tuff which lies 50 cm below the *Homo erectus* skeleton announced in the accompanying article⁶ (KNM-WT-15000), on the west side of Lake Turkana correlates either with the Kimire Tuff or with the Lower Koobi Fora Tuff; until other tuffs lying lower in the section at the site are analysed it is not possible to decide which. But it is clear that the hominid specimen must lie between 1.55 and 1.65 Myr in age.

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A new form of energy dissipation by a moving object in He II

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Despite the superfluid^{1,2} character of ⁴He below the λ transition temperature, there are two distinct mechanisms by which an object moving through the liquid dissipates kinetic energy: (1) it can scatter^{3–5} the excitations (rotons, phonons, ³He isotopic impurities) that constitute the normal fluid component; and (2) for speeds in excess of the Landau critical velocity⁶ (v_L), it can create rotons³, apparently in pairs^{7,8}. The first process has been studied extensively, mostly by measurements of the zero-field mobilities of positive and negative ions^{3–5,9–11}. The second process, which is much rarer, has also been investigated in considerable detail^{12–14} by studies of the motion of negative ions in isotopically pure ⁴He at elevated pressures. Here, we report an attempt to extend the latter type of investigation to lower pressures. This yields unexpected results that can be accounted for satisfactorily only if we postulate the existence of a third dissipation mechanism.

All the experiments were carried out at temperatures low enough that the influence of process (1) could be ignored. At high pressures, P , the drift velocity, $\bar{v}$, of a moving ion is limited by roton pair creation. In weak electric fields, it is accurately described^{12,13} by

$$\bar{v} = v_L + AE^{1/3} \quad (1)$$

where the constant A can be expressed¹⁵ as

$$A = \left(\frac{5.36e\pi^2 \hbar^3 v_L^2}{m_i k_0^4 |V_{k_0, k_0}|^2 \mu_r} \right)^{1/3} \quad (2)$$

m_i is the ionic effective mass, k_0 and μ_r are the roton wave vector and effective mass and V_{k_0, k_0} is the matrix element, unknown *a priori*, that characterizes the pair-emission process. The experimental technique developed for the determination of $\bar{v}$ in weak electric fields has already been outlined^{16,17} and will be described in detail elsewhere¹⁸. Briefly, a group of ions is field-emitted into a large volume (~ 1.5 l) of isotopically purified liquid ⁴He at ~ 80 mK and their average velocity in traversing

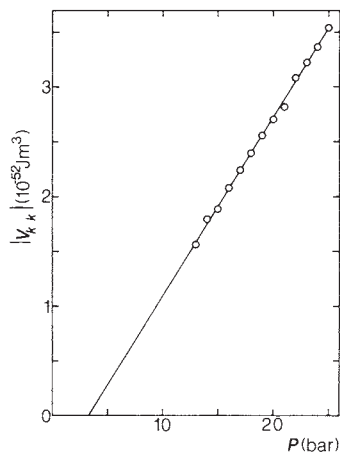


Fig. 1 The modulus of the matrix element $|V_{k_0, k_0}|$, for roton pair creation by negative ions in He II plotted as a function of pressure, P . The solid line has been fitted to the data by the method of least squares.

a region of weak uniform electric field is then measured by means of a time-of-flight technique. Comparison of the experimental $\bar{v}(E)$ data with equations (1) and (2) enables v_L and $|V_{k_0, k_0}|$ to be determined. The values of v_L are found¹⁶⁻¹⁸ to agree to within 1.5% with those calculated from the Landau theory⁶ on the basis of accepted values¹⁹ of the roton parameters. The experimental $|V_{k_0, k_0}|$, incorporating measured²⁰ values of m_i , are plotted as a function of pressure in Fig. 1. It can be seen that $|V_{k_0, k_0}|$ decreases rapidly with falling P and that a linear extrapolation would imply that it became zero at ~ 3 bar. (Metamorphosis of the bare ions to charged vortex rings^{21,22} prevents the extension of this type of experiment much below 13 bar.)

Although there is no theoretical justification for a linear extrapolation *per se*, it seems reasonable to conclude from Fig. 1 that the probability of roton pair creation will become very small at low pressures. One possibility is that V_{k_0, k_0} may pass through zero and change sign at some characteristic pressure. Without an explicit physical model of the roton creation process, one can only guess at the underlying reasons for such behaviour. It seems probable that the most significant result of reducing P is the consequent expansion^{5,20} of the ion; it is conceivable that there may be some critical ionic radius at which the two rotors of a nascent pair will tend to interfere destructively, effectively reducing the probability of the emission event in question.

Regardless of the correctness of such speculations, it is of considerable interest to investigate the behaviour of bare ions moving through He II at low pressures: for low enough temperatures, in isotopically pure ^4He , process (1) can be disregarded; and the data of Fig. 1 strongly imply that process (2) will also be ineffective as a significant source of drag. What, then, will limit the speed of an ion moving under the constant force arising from an external electric field?

An experiment to investigate this question seems at first sight to be precluded by the rapidity with which bare ions undergo the transition to charged vortex rings at low pressures, thereby being lost from the propagating ion cloud whose drift velocity is to be measured. The transition can, however, be suppressed²³ by the application of electric fields $> 2 \text{ MV m}^{-1}$. Measurements in these conditions are not straightforward because of the need to maintain electric fields above this value throughout the whole transit of the ion from source to collector; but they are possible. We have performed a set of measurements of $\bar{v}(P)$ for $E = 2.6 \text{ MV m}^{-1}$ extending down to 1 bar (Fig. 2).

Although the data are subject to considerable scatter, it is immediately evident that the form of $\bar{v}(P)$ is very different from what might reasonably have been anticipated (Fig. 2). As P is reduced below 25 bar, $\bar{v}$ at first increases. This was as expected: first, because of the increase of v_L (dashed line) which causes $\bar{v}$ also to increase (so that, if $\bar{v} - v_L$ were to remain constant, the data would follow the dash-dotted curve); and, second,

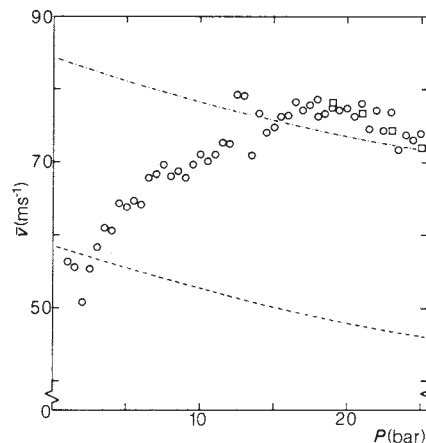


Fig. 2 The drift velocity $\bar{v}$ of negative ions in He II at 0.3 K under an electric field of 2.6 MV m^{-1} , plotted as a function of pressure, P . The dashed curve shows the pressure dependence of the Landau critical velocity for roton creation; the dash-dotted curve indicates the form of $\bar{v}(P)$ that would be expected if $(\bar{v} - v_L)$ remained constant as P was reduced from 25 bar. Squares represent data acquired through use of the electric induction technique described previously²² and circles represent measurements made with a modified (single grid) form of the same technique that has been devised to enable the electric field criterion in the text to be satisfied, and which will be described in detail elsewhere.

because of the decrease of $|V_{k_0, k_0}|$ which causes $\bar{v}$ to rise above the dash-dotted line as is indeed observed. This trend is not, however, continued to lower pressures. Rather, $\bar{v}(P)$ passes through a maximum at ~ 16 bar and then falls rapidly to reach a value near 1 bar that is actually below v_L . The latter observation clearly demonstrates that the drag force that limits $\bar{v}$ at the lower pressures cannot possibly arise from any form of roton emission. Therefore, it seems necessary to conclude that there exists some new dissipation mechanism as well as (1) and (2) mentioned above.

The average rate of energy dissipation by the ion may be written as

$$eE\bar{v} = 2\Delta R_2(\bar{v}) + \dot{\epsilon}_v \quad (3)$$

where the first and second terms on the right correspond, respectively, to dissipation through roton emission and through the unknown additional mechanism, Δ is the roton energy gap and $R_2(\bar{v})$ is the roton pair creation rate. Knowing $\bar{v}$, v_L and the matrix element for pair production (Fig. 1) we can calculate^{7,15} the rate of energy loss through roton creation; by subtraction of this quantity from our measured values of $eE\bar{v}$ we then obtain $\dot{\epsilon}_v$. Application of this procedure to the $\bar{v}(P)$ data of Fig. 2 yields the $\dot{\epsilon}_v$ values shown in Fig. 3. The error bars show the effect of a $\pm 5\%$ uncertainty in the matrix element and the random error in the data is indicated by their scatter. It is evident that the additional dissipation mechanism at first intensifies

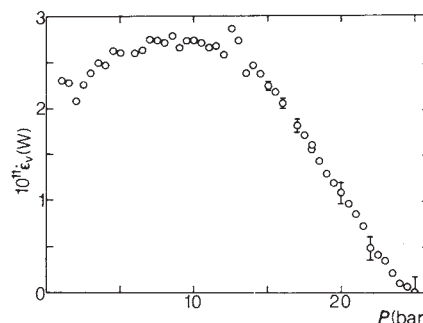


Fig. 3 The rate of energy loss, $\dot{\epsilon}_v$, caused by the new dissipation mechanism for a negative ion moving in an electric field of 2.6 MV m^{-1} . The points, calculated as described in the text from the data in Fig. 2, are plotted as a function of pressure, P . Bars indicate the effect of a $\pm 5\%$ change in the value used for $|V_{k_0, k_0}|$.

slightly with increasing pressure, passes through a maximum at ~ 8 bar and then falls quite steeply, approaching zero near 25 bar.

We propose that a probable candidate for this unknown source of drag lies in the continuous production of microscopic quantized vortex rings. That is, rather than undergoing the well-known transition to a charged quantized vortex ring, the ion creates a succession of elemental rings to which it does not bind. Such a phenomenon is anticipated²⁴ on the basis of Schwarz and Jang's symmetrical creation model^{25,26} of the transition; it could conceivably also occur for the alternative vortex loop model²⁷ if the elemental loop were to become unstable in very high electric fields. We infer that the process can happen at all pressures but that it does so much more weakly at the higher values of P , mainly because of the opposing pressure dependences of v_L and the critical velocity v_c for vortex nucleation. The existence of such a phenomenon would also provide a plausible explanation for the observed small departures¹⁴ of $\bar{v}(E)$ from the behaviour predicted by the roton pair emission theory for large E at 25 bar.

Two specific points arise concerning the relationship of our present results to the large body of work on the interaction of ions with vortices in He II. First, it is well established that, for non-zero temperatures, there is a finite probability of an ion escaping from the vortex on which it is trapped, as the result of a thermal fluctuation²⁸⁻³⁰. In some conditions, therefore, the ion can proceed through the liquid in a series of distinct epochs such that it exists for part of the time as a bare ion and for the rest of the time forms part of a charged vortex ring³¹⁻³⁶. Initially, the ion accelerates under the influence of the applied electric field, and provided that its velocity $v \geq v_c$, the transition to a charged vortex ring may occur. The ring grows and slows and, eventually, particularly for strong electric fields, the ion may escape from the ring through a thermal fluctuation^{31,35,36}; it then starts to accelerate again, and so on. (In those cases where the electric field is inadequate to maintain the stability of the ring against process (1), but still sufficient to cause $v \geq v_c$, the ring shrinks, accelerates and disappears, again liberating the trapped ion^{32,34}; the process then repeats, as before.) The mechanism envisaged here is distinctly different in character, however, in that charged vortex rings as such are not created because trapping of the ion on the nascent ring does not occur: the 'moving object' therefore maintains its continuity of existence as a bare ion. Second, the notion that a moving ion might be able to create a sequence of uncharged microscopic vortex rings was proposed several years ago by Huang and Olinto³⁷. Their suggestion related to a theoretical explanation for the Careri mobility steps which, unfortunately for the theory, were subsequently shown to have been, if not an artefact, then some classical phenomenon quite unconnected with the quantized vorticity of the superfluid under consideration. But, although incorrect in its original context, their proposal can provide a basis for the interpretation of the present experiments.

We may interpret Fig. 3 in the following way. The rate of energy loss to vortex rings can be written

$$\dot{E}_v = \nu E_{\text{ring}} \quad (4)$$

where ν is their nucleation rate and E_{ring} is their average energy. The dissipation rate through this process becomes very small at large P because, for any given velocity, rotons are then more strongly emitted: this is partly a result of the decrease of v_L , partly because of the increase in $|V_{k_0, k_0}|$ and partly as a result of the increase of v_c . The dissipation rate also exhibits a slight decrease at low P , mainly because the ion is travelling more slowly, which is attributable to the decrease of v_c with falling P . The data in this range seem to favour the Muirhead *et al.* theory²⁷ of vortex nucleation slightly over that of Bowley²⁶, for which the predicted pressure dependence of v_c is weaker.

The situation is, however, complicated, first, because of the changes to be anticipated in E_{ring} owing to the pressure dependence of the ionic radius and, second, because of our ignorance of the distribution function of ionic velocities in these conditions. The vortex nucleation rate itself cannot, of course, be evaluated without a knowledge of E_{ring} ; but, if we make the plausible

assumption that the radius of the ring will be ~ 2 nm, slightly larger than the radius of the ion, so that $E_{\text{ring}} \approx 5 \times 10^{-21}$ J, we find from equation (4) that $\nu \approx 4 \times 10^9 \text{ s}^{-1}$ at zero pressure. The latter value seems physically reasonable.

In conclusion, it seems that a third form of drag exists on a moving object in He II, and we suggest that it corresponds to the production of a stream of quantized vortex rings.

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Image distortion near galaxies: dwarfs or lensing?

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Gravitational lensing is a useful tool for studying the mass distribution in galaxies. The average gravitational lens distortion of background galaxy images by foreground galaxies is an independent non-kinematical measurement of galaxy mass distribution M/r (ref. 1), where M is the mass of the galaxy and r its radius. Using an indirect calculation involving estimated luminosity functions and absolute magnitudes, Philipps² recently claimed that our measurement of field galaxy mass distribution by galaxy-galaxy gravitational lensing was contaminated by large numbers of foreground dwarf galaxies. I present here a direct measurement of this effect: the cross-correlation between these samples clearly reveals clustering of foreground dwarf galaxies, with an amplitude slightly less than that given by our original simulation¹. Thus, this effect does not change our conclusions regarding the average galaxy mass distribution.

Galaxy mass can be expressed as an equivalent orbit velocity at some radius. The upper limit we obtained for the equivalent

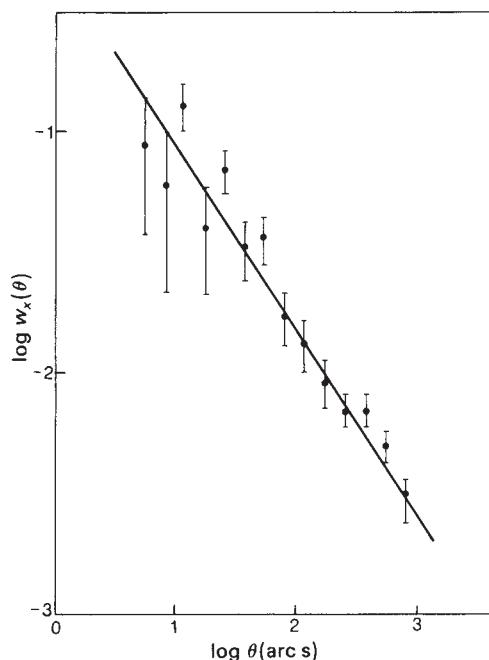


Fig. 1 Dwarf galaxies clustering about bright galaxies. The two-point cross-correlation function is plotted for the foreground galaxies and background galaxies used in the determination of galaxy mass distribution by gravitational light deflection¹. Note that contamination of the background galaxy data set by sub-luminous clustered foreground galaxies is small ($\sim 10\%$) at 10 arc s angular separation between the bright and faint galaxies. Even-odd bin round-off effects at small separation angle have not been corrected. The amplitude of this correlation function is 12 times less than that predicted by Philipps², and is in reasonable agreement with our simulation.

circular velocity, while small compared with some heavy halo models, is consistent with dynamically derived estimates for a sample of galaxies of all types¹. For example, for a mean mass cut-off radius of $65 h^{-1} \text{ kpc}$, our 3σ upper limit for the equivalent circular velocity $\langle v_c \rangle = [(GM)/r]^{1/2} = 190 \text{ km s}^{-1}$. [h is the value of the Hubble parameter in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$]. The search for a statistical gravitational-lens image distortion of background galaxy images about the positions of foreground galaxies is complicated by several low-level systematics. We obtained upper limits to many systematics by substituting either random positions or stars for the foreground or background galaxies. Remaining contaminating effects, which are largest at small angular separations, must be modelled, simulated or, even better, measured directly.

Our foreground sample of galaxies has magnitudes in the range 19–21.5 J mag. Our background sample covers 22.5–23.5 J mag. A fraction (originally estimated¹ at 15%) of our 'background' galaxies are actually sub-luminous galaxies physically clustered with our foreground galaxies. This effect is one of several known systematics of which we were aware and which are corrected¹. However, a direct measurement is always better than a calculation.

The average magnitude of galaxies in our foreground sample is 20.75 J mag and that of our background sample is 23.06 J mag. Thus, any physical companions of the foreground galaxies, appearing as background galaxies, have absolute magnitudes about 2 J mag fainter than the average foreground galaxy in our sample.

I can test the hypothesis of significant dwarf galaxy contamination of our background sample by direct internal analysis of our data, without reference to any assumed luminosity function. Philipps claims that large numbers of dwarf galaxies clustering about our foreground galaxies dilute our true background sample by a factor of 4–1.5 over the range 4–40 arc s separation angle, giving a cross-correlation function³ $w_x(\theta) = 9\theta^{-0.8}$, where

$w_x(\theta)$ is the excess probability, over random, of finding a faint galaxy near a bright galaxy, separated by an angle θ . This prediction by Philipps is a necessary condition for his conclusion; that is, if $w_x(\theta)$ for our observations falls significantly below $9\theta^{-0.8}$, for whatever reason, this hypothesis fails.

Taking the same foreground and background galaxy data sets used in our galaxy mass determination¹, and forming the two-point cross-correlation function, I obtain the correlation shown in Fig. 1. There were 1.3×10^7 galaxy pairs in the cross-correlation, with average magnitudes of 21.04 and 23.06 J mag in the foreground and background samples, respectively. The best-fit line has the same slope (-0.77) found in our galaxy autocorrelation functions. I obtain (3σ):

$$w_x(\theta) = (0.7 \pm 0.1) \theta^{-0.77} \quad (1)$$

This is a factor of 12 below Philipps' estimate, and is close to our original¹ estimate, based on a Monte-Carlo simulation using a Schechter luminosity function. In the range of θ that dominates our gravitational lens distortion measurement ($0.7 < \log \theta < 1.5$), our measured $w_x(\theta)$ averages $< 10\%$. Our original plots of image distortion were corrected for 15%. Thus, our equivalent circular velocities were overcorrected by 5%, and the effect of our new calibration of this effect is slightly to enhance our null result, the conclusions of our paper being unchanged. The formal discrepancy between Philipps' estimate and our observations is 38 s.d. Thus, intrinsically faint galaxies do not significantly contaminate our background sample. Below I briefly discuss the apparent origin of Philipps' anomalously large amplitude.

To estimate this contamination effect indirectly, we require the luminosity function in some standard system, and then we must transfer J magnitudes of average and dwarf galaxies onto that system. Using $B = J + 0.23(B - V)$, $B - V = 0.8(J - F)$ and the mean $B - V$ colours of average and dwarf galaxies at 21 J mag (1, 0.3), I obtain $B_{fg} = 21.2$ mag and $B_{fg} = 23.0$ mag for the mean apparent B magnitudes of these two galaxy type samples in our foreground sample. The colour-dependent K -corrections at $J = 21$ mag ($z = 0.2$) will be different for the dwarf and average galaxy samples. I estimate the difference in the colour terms as $\Delta K = 0.8$ mag. Since $B - M_B = 25 - 5 \log h + 5 \log r + K$, I obtain $M_{Bd} - M_B = 2.4$ mag for the difference between the dwarf and average galaxy absolute magnitudes in our foreground sample. If $\langle M_B \rangle = -20$, then $\langle M_{Bd} \rangle = -17.6$ mag.

Historically, published values for the galaxy luminosity function, without regard for the depth of the sample, have varied widely. Philipps chose the value $0.05 h^3 \text{ Mpc}^{-3} \text{ mag}^{-1}$ from a range of observed values⁴. Recent deeper and more statistically homogeneous galaxy samples^{5–7} have given differential luminosity functions at $M_B = -17.6$ mag ranging from 0.006 to $0.015 h^3 \text{ Mpc}^{-3} \text{ mag}^{-1}$. Using $0.01 h^3 \text{ Mpc}^{-3} \text{ mag}^{-1}$, Philipps' result would be within a factor of two of our measurement, well within the errors of his estimate. Felten⁸ argues for even lower values for higher redshift samples of field galaxies. Another theoretical estimate for this effect, which would predict negligible contamination for our sample, can be found in ref. 9, Table 4.

In this instance, due to uncertainties in published galaxy luminosity functions, a direct measurement of the two-point cross-correlation function of our foreground and background galaxy samples is more reliable than any calculation based on assumed luminosity functions and K -corrections. Given some foreground galaxy redshifts, cross-correlations in these faint galaxy samples may eventually yield new data on the faint end of the galaxy luminosity function.

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Intermittent baroclinic instability and fluctuations in geophysical circulations

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The presence of blocking highs in atmospheric circulation, bringing periods of stable weather alternating with more variable weather patterns, is an example of two distinct modes of behaviour occurring under similar conditions in a natural system. The occurrence of two or more different flow regimes under a given external forcing is a consequence of nonlinear dynamics that allows the system to equilibrate in different modes. Each mode can be stable over a range of conditions and yet there may be sudden transitions between modes. We have now discovered in a laboratory experiment using rotating fluids a case of at least two modes of behaviour under identical conditions. In one mode, a weak continuous forcing drives a mean circulation that is steady but on which are superimposed waves whose amplitudes vacillate under the influence of viscous dissipation. Under suitable conditions there is an alternative mode in which the mean flow fluctuates and unstable waves occur only in short bursts at periodic intervals separated by relatively long periods of stable flow with no waves. The existence of multiple equilibrium states is of interest in the climatology of all geophysical and astrophysical fluid bodies. Our discovery of a mode involving intermittent bursts of instability and long-period fluctuations of mean flow (resulting purely from internal dynamics rather than from variations in external forcing) may have implications for the variability of oceanic circulation or solar radiation on timescales of a few years to centuries.

The condition of solid-body rotation (or zero motion relative to a rotating reference frame) can never be attained by a fluid containing gradients of temperature or chemical composition¹. This is because diffusion of density across the curved surfaces of constant density drives a meridional (Eddington–Sweet) circulation in planes parallel to the axis of rotation^{2,3}. Conservation of angular momentum of the circulating fluid in turn leads to an even larger azimuthal flow with an associated vertical shear and finite inclination of surfaces of constant density relative to surfaces of constant pressure. This azimuthal flow can, under suitable conditions, be unstable to perturbations that convert gravitational potential energy into kinetic energy of waves or of irregular eddy motions superimposed on the mean circulation⁴; this mechanism for conversion of energy is termed baroclinic instability.

In our experiments, a cylindrical container of radius $R = 30$ cm and with a planar base perpendicular to gravity, rotates

with angular velocity Ω about an axis through its centre and parallel to gravity. A layer of water 10 cm deep is allowed to approach solid-body rotation before a dilute sugar solution ($0.5\text{--}5 \times 10^{-3} \text{ g g}^{-1}$) is slowly released onto the base from a small tube against the wall. Once a lower layer has accumulated to a depth of 10 cm, the pipe is removed and the top of the container sealed to prevent surface stress and evaporative cooling. Vertical motions of the density interface are detected by conductivity probes on the axis of rotation and at a radius of 20 cm. Each probe consists of a platinum wire electrode suspended vertically through the interface⁵. A small amount of salt is mixed with the upper layer before filling to produce a conductivity contrast between layers and the circuits are completed through a flat plate electrode on the wall of the container.

The vertical position z of the density interface on the axis of rotation is a convenient measure of the azimuthal circulation. In fact, by considering conservation of momentum and volume in each layer, it can be shown that variations of z at the centre of the cylinder are caused primarily by variations of a horizontally averaged velocity difference between layers (the radial pressure gradient resulting from the sloping interface being everywhere in approximate balance with Coriolis forces). Waves are not detected at the axis when the flow is unstable because they have a node at the centre and a maximum amplitude somewhere near the wall. On the other hand, conservation of volume implies that mean circulations have little influence on interface height 20 cm from the axis, where the second probe is located, so that the latter detects only waves and eddies.

After filling and a period during which relative motions induced by the filling procedure decay, diffusion of sugar across the thin interface begins to have a significant effect. Baroclinic instability of the resulting azimuthal flow depends on the internal Froude number $F = 4\Omega^2 R^2 / (g(\Delta\rho/\rho)h)$ (h is the depth of both layers, g the gravitational acceleration and $\Delta\rho/\rho$ the fractional density difference), the influence of friction expressed in terms of the Ekman number $E = \nu/2\Omega h^2$ (ν is the kinematic viscosity) and the Rossby number $Ro = \Delta U/2\Omega R$ (ΔU is a characteristic velocity difference^{4,5}). Waves are predicted to grow when $F > 0(10)$ and $E^{1/2}Ro^{-1} < 0(1)$. In the present system, the velocity difference ΔU is a function of solute diffusivity, interface thickness and curvature of density surfaces (or $\Omega^2 R/g$). The above criterion, using a velocity scale measured from floating markers, predicts instability for all experiments in which the outer probe detected the growth of waves. As predicted, no waves appeared in experiments for which $F < 10$. Although the Eddington–Sweet circulation driven by solute diffusion has been detected previously in laboratory experiments⁶, its instability has not.

Other phenomena detected in our experiments are wave amplitude vacillation and the influence of instability on the mean flow. In Fig. 1 the waves persist with a mean amplitude that remains nearly constant over long times, apart from obvious amplitude vacillations that are a common feature of finite amplitude baroclinic waves^{5,7–10}. At the same time the mean azimuthal circulation is practically invariant, implying a balance between spatially averaged dissipation and forcing over all timescales.

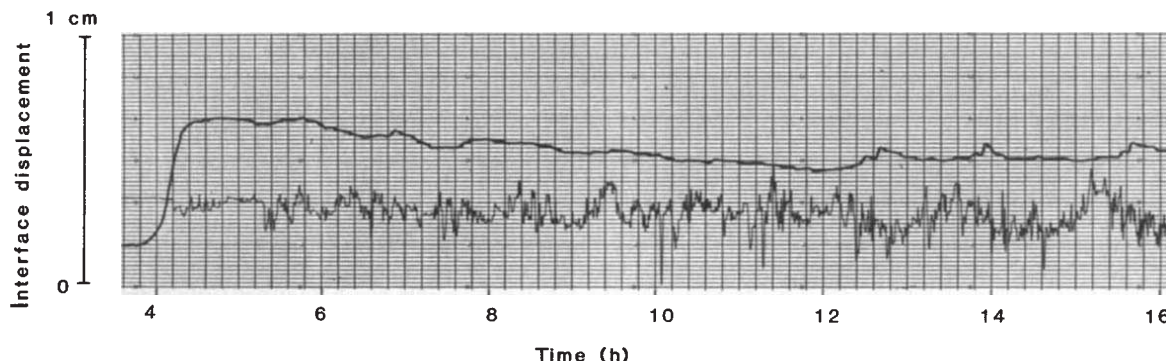


Fig. 1 Vertical displacements of the density interface as functions of time after filling as detected by conductivity probes at the axis of rotation (heavy line) and at a radius of 20 cm (lower trace) for an experiment in which $\Omega^2 R/g = 4 \times 10^{-2}$, $E = 8 \times 10^{-5}$, $Ro = 0.03$ and $F = 76$; $\Omega = 1.2 \text{ rad s}^{-1}$, giving a rotation period of 5.2 s. Baroclinic waves are continuously present and the mean flow is essentially steady.

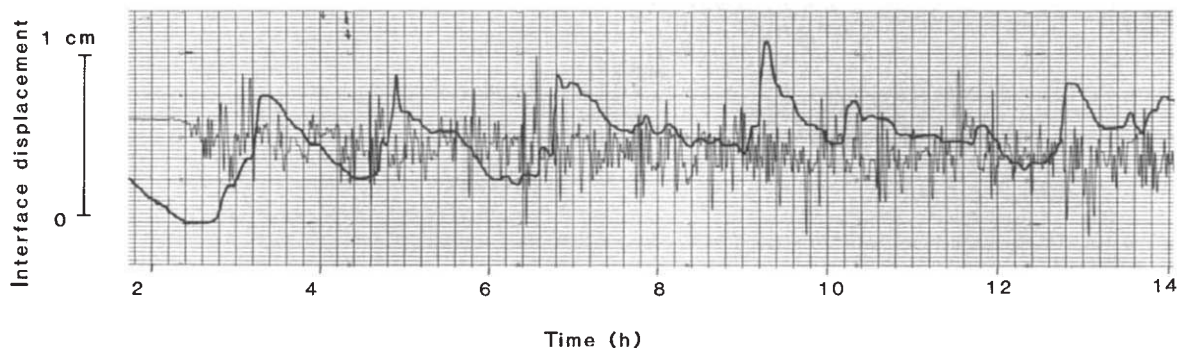


Fig. 2 Vertical displacements of the density interface in an experiment similar to that of Fig. 1 but with $F \approx 150$. Rotation period, 5.2 s. Baroclinic waves are continuously present (light trace) but induce fluctuations in the mean flow (heavy line).

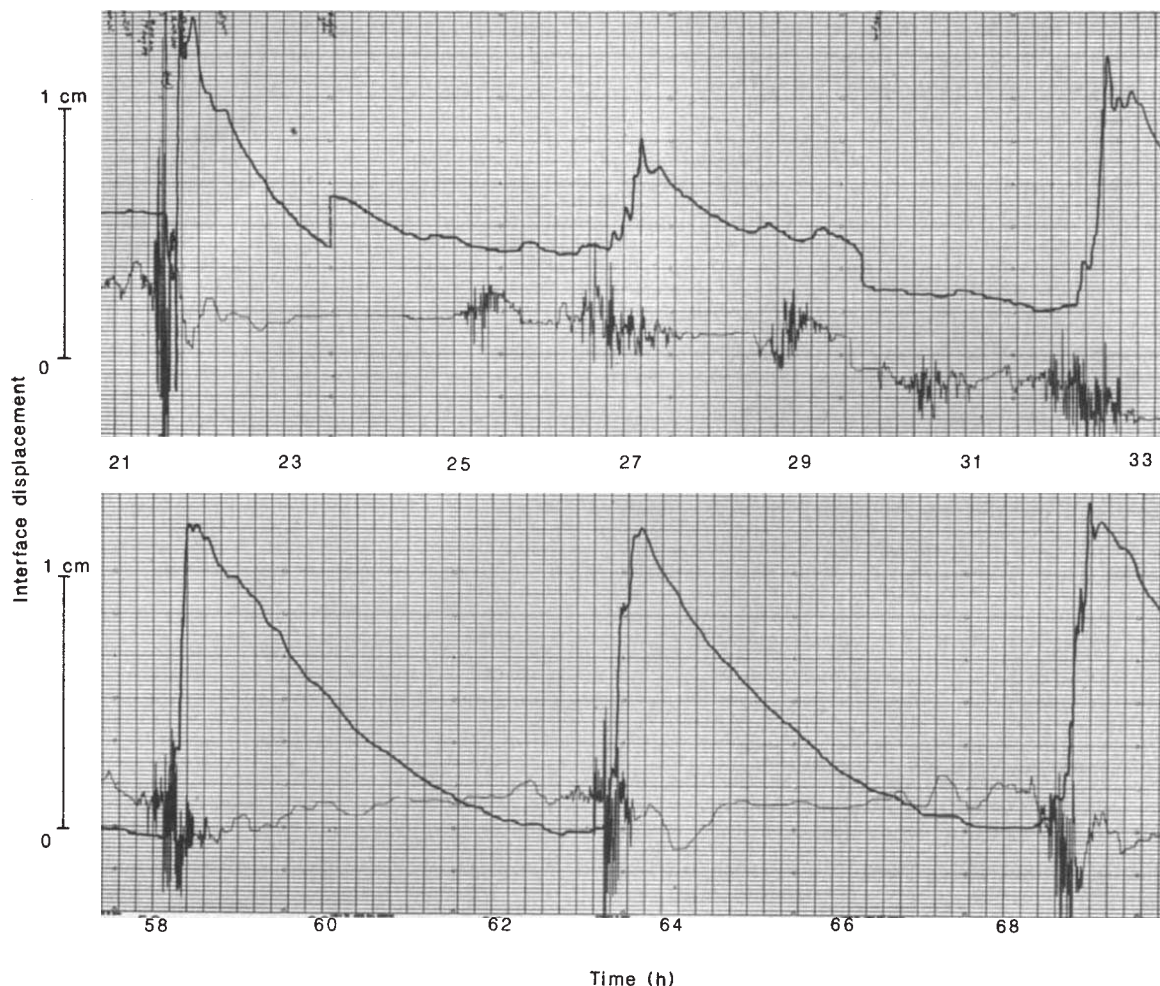


Fig. 3 Displacements of the density interface during two separate periods for the experiment of Fig. 1, showing the effect of an 8% increase in Ω at 21.6 h after filling. Baroclinic waves appear only intermittently (light trace) and the mean circulation (heavy line) eventually settles into a period oscillation. In this experiment, the periodic oscillations persist for 80 h until diffusion has so altered the density distribution that waves dies out.

When the experiment is repeated with the density difference halved, waves are again continuously present (Fig. 2). However, they show larger fluctuations of amplitude, and there are marked fluctuations in the mean azimuthal flow. In this case it seems that the forcing and dissipation are not in balance even over timescales up to hundreds of rotation periods.

The interaction of unstable waves with the mean flow can give rise to two flow regimes in a given experiment. A transition can be achieved by inducing a small instantaneous increase in

the angular velocity of the container. Figure 3 shows the result of increasing Ω from 1.2 rad s^{-1} to 1.3 rad s^{-1} in the experiment of Fig. 1. Immediately after Ω is changed, the lower layer is spun out towards the wall by a bottom Ekman layer and the interface falls at the axis of rotation. Within a couple of rotation periods (10–20 s), waves attain very large amplitude and can be observed when looking horizontally through the container. Such observations, as well as closed-circuit video monitoring of markers floating on the free surface, reveal that closed eddy

circulations form as the waves reach maximum amplitude. Waves and eddies then redistribute mass and angular momentum across the radius, reducing vertical shear and lifting the interface at the axis to well above its initial height. Without a sufficient interfacial shear to sustain them, the waves and eddies are rapidly dissipated by Ekman suction over a time of 10^2 rotation periods. However, isobaric surfaces must still be curved and diffusion again proceeds to slowly build up a mean azimuthal flow and vertical shear. The upper layer motion is clockwise relative to the container, which rotates in the anticlockwise sense. Hence the flow increases the curvature of density surfaces and further stimulates forcing due to the Eddington–Sweet mechanism.

After a period during which the system settles down, the flow in the experiment of Fig. 3 is stable for long periods of time (~ 5 h or 4,000 rotation periods), but is punctuated at regular intervals by relatively short bursts (lasting ~ 400 rotation periods) of unstable waves and eddies. Instability seems to occur whenever the vertical shear exceeds a magnitude beyond which release of potential energy by azimuthal waves overcomes dissipation of perturbations (E^3/Ro becomes small enough). It then causes a rapid readjustment of the mean circulation (taking as little as 150 rotation periods in the case shown). The cycle is repeated for as long as there is a sufficiently strong forcing: both baroclinic waves and long-period oscillations of the mean flow die out only when the interface becomes very thick and concentration gradients very small. The shortest period for oscillations of the mean flow found in any experiment is 1,100 rotation periods, although a wider range of conditions needs to be investigated.

The different rotation rate is not in itself significant in those experiments perturbed by a small change in Ω , as experiments that begin with the higher value of Ω but with other parameters equal also behave in the manner shown on Figs 1 and 3, with waves persisting in a continuous fashion until a change in Ω is impressed on the system. Thus, two flow regimes can occur under a given set of parameter values and in a given experiment. The regime realized must be dependent on the initial conditions or history of the flow.

Both the multiplicity of flow regimes and the possibility of energy release in short periodic bursts may be of relevance to circulation and heat transport through radiative zones in stellar interiors, where Eddington–Sweet circulations caused by thermal gradients have been predicted to be significant^{11,12}, but where only the stable and continuously unstable flow regimes have been considered. The possibility of a regime involving large fluctuations in mean circulation on timescales of the order of 1,000–5,000 rotation periods suggests the possibility of periodic variations of solar energy output on scales of tens to hundreds of years (using a rotation period for the Sun of 25 days). In oceanic circulation, the effects of other energy sources such as surface wind stress and meridional temperature gradients are expected to dominate over those of Eddington–Sweet circulations. However, on the scale of ocean basins, these forces too drive unstable baroclinic flows⁴ in which the interaction of nonlinear instability and mean flow might lead to multiple flow regimes or circulations fluctuating on long timescales. Again, if forcing and dissipation are such that mean flow fluctuations do occur, the oscillation period must scale with the rotation period of the system and our experiments indicate timescales of 3–15 yr. Data on the intensity of western boundary currents are insufficient to test for such fluctuations.

Garnet phase of MgSiO_3 , filling the pyroxene–ilmenite gap at very high temperature

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Melting experiments on MgSiO_3 , the major constituent of the Earth's mantle and planetary interiors, have now been conducted at a static pressure of 20 GPa. These experiments reveal the presence of a new garnet-like non-cubic phase of MgSiO_3 , which melts congruently at 2,250 °C. This new silicate phase and its stability have significant implications for mantle mineralogy, chemical fractionation by partial melting and also for the occurrence of aluminium-deficient cubic garnet (majorite) in shocked meteorites. These high-pressure experiments at temperatures $>2,000$ °C have been made possible by the development of a large-volume high-pressure device at Nagoya University^{1–4}.

The high-pressure and high-temperature experiments were carried out using an MA8-type high-pressure apparatus^{1–4} and were made under a fixed ram load, which generated a pressure of 20 ± 2 GPa. To generate a very high temperature constant enough to melt silicates in a high-pressure cell, a composite of tungsten carbide and diamond was used for the heating element. The run temperature was measured by a W/W26%Re thermocouple, the hot junction being in contact with the graphite sample capsule. A powder of synthetic orthoenstatite was used for the starting material. Heating was limited to a few minutes to avoid contamination of the run product with the pressure medium, which was made of sintered MgO. Thin sections for observation by microscope and electron microprobe were prepared from the samples (~ 1 mm³), which were recovered after isobaric quenching following pressure release.

The run product at 1,760 °C, which show a granular texture indicating that recrystallization occurred in the solid state, can be identified by powder X-ray diffraction as the ilmenite phase of MgSiO_3 . The run product at 2,000 °C shows a more finely grained texture and consist of crystals with very low birefringence. The preservation of the starting composition can be

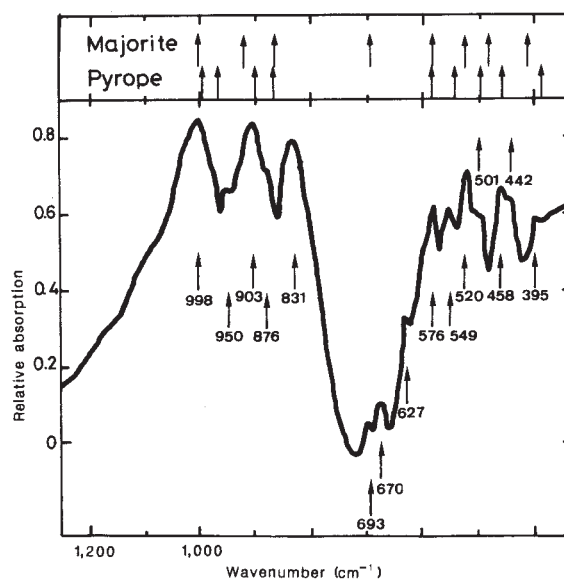


Fig. 1 Infrared absorption spectrum of non-cubic garnet of MgSiO_3 (1.8-mg sample in 190-mg KBr). The arrows at the top indicate the peak positions for majorite observed by Jeanloz¹¹ and for pyrope garnet observed by Moore¹². The present data show a similarity to those of two cubic garnets except for the presence of mode splitting.

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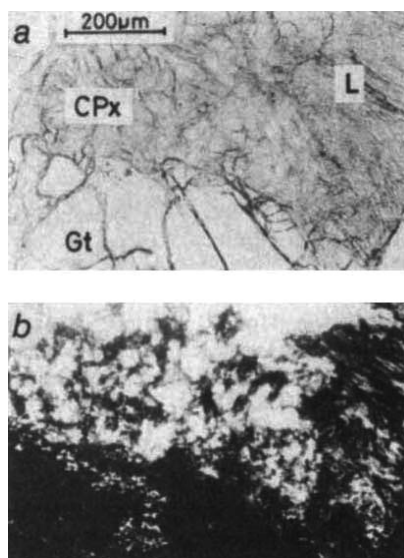


Fig. 2 Photomicrographs of the run product at 2,250 °C. *a*, Open nicol; *b*, crossed nicols. L, liquid phase; CPx, clinopyroxene; Gt, non-cubic garnet.

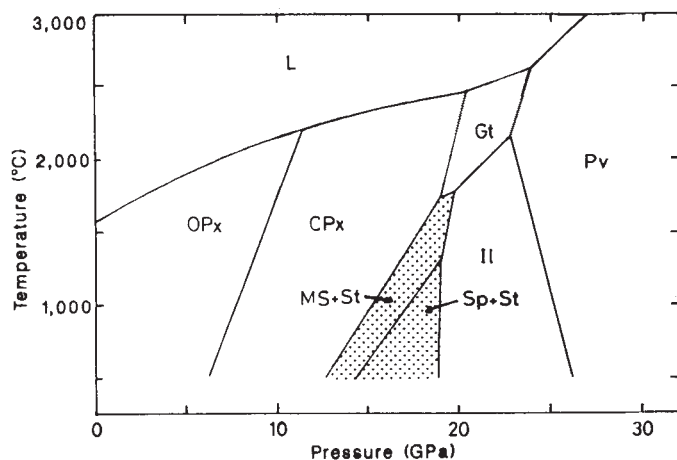


Fig. 3 Phase diagram for the composition of MgSiO_3 . The stippled region is the pyroxene-ilmenite gap in polymorphic transition in MgSiO_3 . The melting curve is drawn after ref. 17 below 5 GPa, and is extrapolated to higher pressures. OPx-CPx boundary is after Kanzaki (unpublished data). Il-Pv boundary is after ref. 20. Other phase boundaries are arbitrarily drawn to fit the experimental observations¹⁸⁻²⁰ and phase rule.

confirmed by electron microprobe analysis, which indicates $\text{MgO} = 50.5 \pm 1.0$; $\text{SiO}_2 = 49.5 \pm 1.0$; $\text{Al}_2\text{O}_3 < 0.1$ in mol%. The pattern of powder X-ray diffraction generally fits that of pyrope garnet (ASTM 15-742), although the peaks shift towards a low diffraction angle and some of them show splitting. A garnet phase with a low crystallographic symmetry has been reported in some silicates and germanates, for example, high-pressure polymorphs of MnSiO_3 (ref. 5) CdGeO_3 (ref. 6) and CaGeO_3 (ref. 7) with tetragonal symmetry. The observed peak splitting indicates that the present garnet-like non-cubic phase (referred to hereafter as non-cubic garnet) is probably isomorphous to tetragonal MnSiO_3 , and different from majorite, an aluminium-deficient cubic garnet found in shocked meteorites^{8,9}. The powder X-ray diffraction data for the non-cubic garnet of MgSiO_3 are tentatively classified as having tetragonal symmetry (Table 1). The mode splitting is so small that detailed assignment may involve some ambiguity; the uncertainty in lattice parameters is of the order of $\pm 0.01 \times 10^{-10}$ m. Sawamoto and Kozaki¹⁰ report lattice parameters of $a = 11.472 \times 10^{-10}$ m, and $c = 11.398 \times 10^{-10}$ m, and suggest that the distortion of cubic garnet of MgSiO_3 to non-cubic phase is associated with a density

Table 1 Powder X-ray diffraction data for MgSiO_3 garnet

Intensity	d_{obs} (10^{-10} m)	d_{calc} (10^{-10} m)	<i>hkl</i>
		4.685	211
10	4.668	4.668	112
		3.069	321
10	3.061	3.064	312
5	3.045	3.056	213
65	2.870	2.873	400
35	2.846	2.852	004
100	2.566	2.569	420
		2.565	402
50	2.553	2.554	204
		2.446	332
60	2.441	2.442	323
30	2.343	2.343	422
20	2.334	2.334	224
30	2.252	2.253	431
		2.248	413
30	2.243	2.243	314
		2.253	510
		2.253	501
		2.238	105
		2.097	521
10	2.096	2.096	512
5	2.082	2.085	215
		2.031	440
		2.024	404
20	1.8631	1.8637	611
10	1.8499	1.8510	116
10	1.8178	1.8169	620
		1.8510	602
		1.8048	206
20	1.6539	1.6545	444
20	1.5921	1.5935	640
25	1.5912	1.5899	604
20	1.5849	1.5853	406
35	1.5359	1.5347	642
50	1.5334	1.5323	624
35	1.5301	1.5282	426

$$a = 11.491 \times 10^{-10} \text{ m}, c = 11.406 \times 10^{-10} \text{ m}.$$

increase of about 1 %. They are now trying to synthesize large single crystal samples for more accurate characterization of the crystallography.

Infrared spectral measurements of this phase and the result are plotted in Fig. 1 with the peak positions reported by Jeanloz¹¹ for natural majorite, $(\text{Mg}_{0.79}, \text{Fe}_{0.21})\text{SiO}_3$, and those reported by Moore *et al.*¹² for natural pyrope garnet. The data indicate mode splitting in this low-symmetry garnet.

Figure 2 shows a photomicrograph of the run product at 2,250 °C, in which clinopyroxene and non-cubic garnet are identified by powder X-ray diffraction. Figure 2*b* consists of non-cubic garnet crystals and Fig. 2*a* of clinopyroxene crystals. Both minerals show granular texture, while the right-hand side consists of a clinopyroxene and non-cubic garnet assemblage with a fine fibrous texture, which indicates quenching from the melt at high pressure. The electron microprobe analysis shows the same composition ($\text{MgO} = 50.4 \pm 1.0$, $\text{SiO}_2 = 49.0 \pm 1.0$, $\text{Al}_2\text{O}_3 = 0.6 \pm 0.3$ mol%) in these three phases (clinopyroxene, non-cubic garnet and melt). A little contamination during heating by H_2O and dopants from the pressure medium material has been found in the present system by Kato and Kumazawa⁴. Contamination of alumina is crucial in synthesizing the Al_2O_3 -free garnet. However, its content is so small that the phase stability may not seriously be affected. A small amount of H_2O should have little effect on the melting mode in this system. The above observations indicate that the experimental conditions (20 GPa, 2,250 °C) are very close to the triple point of clinopyroxene, non-cubic garnet and isochemical melt. Above this triple point, MgSiO_3 melts congruently at higher pressures.

The presence of alumina-deficient iron-magnesium garnet (majorite) in shock-metamorphosed meteorites has indicated

the possibility of its formation in pure MgSiO_3 garnet at high pressures and high temperatures. Ringwood and Major¹³ have reported the synthesis of garnet with a composition of $\text{Fe}/\text{Fe} + \text{Mg} = 0.25$ and $\text{Al}_2\text{O}_3 = 1.3$ mol % at 1,000 °C and above 25 GPa. Akaogi and Akimoto¹⁴ found that, in the enstatite (MgSiO_3)-pyrope ($\text{Mg}_3\text{Al}_2\text{Si}_3\text{O}_{12}$) system below 1,200 °C, at least 5 mol % Al_2O_3 is required to stabilize garnet. We have confirmed the expected formation of alumina-free garnet of MgSiO_3 at much higher temperatures than the previous studies. The synthesized garnet is not cubic but of lower symmetry. In some meteorites, cubic majorite garnet occurs in association with glass and high-pressure phases of orthosilicate (spinel and modified spinel)¹⁵, suggesting that crystallization involves melting at high pressures. This phase assemblage suggests that pressure and temperature conditions of majorite formation are close to those of the present experiment. The only difference between majorite garnet and the present non-cubic phase is the composition of $\text{Fe}/\text{Mg} + \text{Fe}$. Therefore, we anticipate the future finding of non-cubic aluminium-free garnet instead of cubic majorite in shocked enstatite chondrites (E) and bronzite chondrites (H), which have low $\text{Fe}/\text{Mg} + \text{Fe}$ in silicate phases. The detailed relationship between the cubic and non-cubic garnets is an important problem to be studied in order to clarify quantitatively the conditions prevailing in majorite-bearing meteorites.

The pressure-induced phase transitions in magnesium silicates (Mg_2SiO_4 and MgSiO_3) have been extensively studied because of their importance for investigating the nature of the Earth's mantle¹⁶. The transition in MgSiO_3 at ~1,000 °C is: orthopyroxene (OPx) → clinopyroxene (CPx) → modified spinel (MS) + stishovite (St) → spinel (Sp) + St → ilmenite (Il) → perovskite (Pv), and it possesses a gap between CPx and Il in polymorphic transition. In contrast, the transition in Mg_2SiO_4 shows a simple mode: two steps of polymorphic transition followed by a disproportionation reaction: olivine → MS → Sp → Pv + St. The disproportionation reaction of CPx involving stishovite, followed by the recombination reaction to Il, is poorly understood. The stability field of the new non-cubic garnet borders those of CPx and Il, all in the polymorphic relation, and the non-cubic garnet fills the gap between CPx and Il in the polymorphic transition of MgSiO_3 at high temperature. Therefore, MgSiO_3 has five polymorphic phases, OPx → CPx → Gt* → Il → Pv, in continuous sequence with increasing pressure (Gt* denotes non-cubic garnet). The presumed phase relation for MgSiO_3 composition is shown in Fig. 3, which summarizes the latest experimental data¹⁷⁻²⁰. The two phase assemblages involving stishovite (stippled area in Fig. 1) at the lower temperatures may be interpreted as a result of the abnormally high density and low entropy of stishovite.

We note several important implications of the present result for the constitution of the Earth and planetary interiors. The congruent melting of MgSiO_3 persists continuously from pyroxene to perovskite through non-cubic garnet, so that the pyroxene-stoichiometric phase remains as a major solid phase in the partial melting process throughout the mantle, regardless of the alumina and other minor elemental concentrations of the candidate material. Because the density of garnet is expected to be larger than the silicate melt throughout the upper mantle²¹, it may sink, thereby constituting the bottom of the upper mantle. The fractionation of garnet could have an important role in the formation of the layered structure in the mantle.

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Neogene history of the calcite compensation depth and lysocline in the South Pacific Ocean

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The depth-time pattern of calcite accumulation recorded at Deep Sea Drilling Project (DSDP) sites beneath the oligotrophic subtropical gyre in the south-east Pacific Ocean allows us to define here both the calcite compensation depth (CCD) and the lysocline. The Neogene CCD history is one of shoaling before 15 or 20 Myr and subsequent deepening to the present level of 4,100 m. A broad zone of increased calcite dissolution, 600 m thick, formed during early to middle Miocene time and has remained. This zone, the lysocline, denotes an important change in ocean chemistry and may reflect either an increase in the volume of more corrosive deep water beginning about 18 Myr and reaching steady state by 14 Myr, the time of rapid ice build-up in Antarctica, or an increase in calcite rain rate caused by an increase in surface-water productivity.

Early workers in marine geology realized that throughout most of the deep ocean the calcium carbonate microfossil tests generated in surface waters dissolved before they could be incorporated into the sediments¹. Experiments in the Pacific by Peterson² and Berger^{3,4} showed that calcite dissolution did not increase linearly with depth but that little dissolution occurred above about 3,500 m and rapid dissolution occurred at greater depths. This depth zone of rapidly increasing dissolution rate is the lysocline^{4,5} and that depth at which the dissolution rate of calcite matches the supply rate, and below which there is no net CaCO_3 accumulation, is the CCD^{6,7}.

Studies of piston cores⁸ and surface sediments⁹ from the South Pacific have shown the present CCD to be at ~4,100 m depth, and the top of the lysocline near 3,500 m. Heath¹⁰ studied calcium carbonate deposition in the equatorial Pacific and inferred changes in the CCD through Cenozoic time. Berger¹¹ first incorporated seafloor subsidence history into Pacific CCD studies, using an empirical age-depth curve similar to that compiled by Sclater *et al.*¹² Results of this study of equatorial Pacific sediments gave the first reasonable estimate of the equatorial Pacific CCD history. An important event in this history was the rapid deepening of the CCD from ~4,000 to 4,800 m at 35-40 Myr. Van Andel and co-workers used empirical subsidence curves and DSDP data to determine the CCD histories of the equatorial and non-equatorial Pacific¹³⁻¹⁵. Their results refined those of Berger for the equatorial Pacific and

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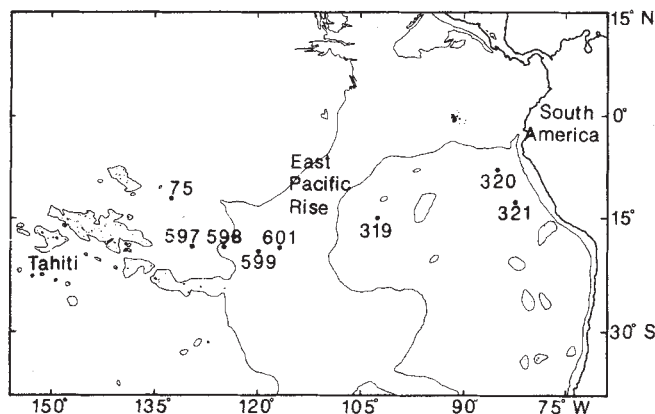


Fig. 1 Index map of the South Pacific showing sites used to construct the subsidence, CCD, and lysocline history of the region.

showed that CCD has been ~600 m shallower (4,000–4,200 m) since the early Oligocene in areas 10° or more north and south of the Equator.

We have determined the history of CaCO_3 deposition in the South Pacific based on data from the site chapters in the Leg 8 (ref. 16), Leg 34 (ref. 17) and Leg 92 (ref. 18) DSDP Initial Report volumes, converting the other data to the Leg 92 biostratigraphy. For each of the eight sites analysed (Fig. 1) we determined intervals of CaCO_3 deposition at rates typical of oligotrophic oceans, intervals over which there was an order-of-magnitude reduction in the mass accumulation rate of CaCO_3 -rich sediments, and the times at which CaCO_3 accumulation stopped¹⁹. The latter is a functional definition of the sedimentary CCD⁹. We presume that the large decrease in rate records the influence of the lysocline. The times of these changes are plotted along the subsidence curves¹⁹ and produce the first reconstruction of separate histories of the CCD and lysocline in the Pacific (Fig. 2).

The eight sites chosen for this study provide a generalized history of the lysocline and the CCD. Site 601 has always been above the lysocline. Sites 598 and 599 are now below the lysocline and above the CCD; Sites 319 and 597 have passed through both the lysocline and the CCD. The two oldest sites, 75 and 321, passed through the CCD abruptly with no evidence of reduced calcite accumulation rates before reaching the CCD. Site 320 was spot-cored; the youngest calcareous sediments recovered there are 15 Myr old. Nonetheless, the reasonably good agreement between the CCD from Site 597 and the maximum age of the transition through the CCD at 320 suggests that these sites passed through the CCD at about the same time.

The CCD in the South Pacific was at ~4,200 m in late Oligocene time, shoaled ~500 to a depth of 3,700 m in the middle-early Miocene, and has deepened since then to the present depth of ~4,100 m (Fig. 2). This history is similar to that determined by van Andel *et al.*¹⁵ for the subtropical and temperate Pacific.

The absence of a zone of decreased preservation²⁰ or reduced CaCO_3 accumulation above the CCD at Sites 75 and 321 suggests that the lysocline was not well developed before 20–17 Myr. It then developed and shoaled to 600 m above the CCD by 13.5 Myr. This 600-m dissolution zone (shaded in Fig. 2) has been present ever since. Linear sedimentation rates for CaCO_3 -rich sediment above the lysocline at Sites 597, 598, 599 and 601 and above the CCD at Sites 75 and 321 are all ~3–10 m Myr⁻¹. Site 319 had higher rates: 11 m Myr⁻¹ just above the CCD and 35 m Myr⁻¹ for older, shallower sediments¹⁹.

All investigations of the Cenozoic history of the CCD have shown a shoaling of the CCD from the middle Oligocene to middle or late Miocene time, followed by a younger deepening, broadly similar to the information shown in Fig. 2^{15,21–23}. A rise in the CCD must reflect some combination of (1) increased CO_2 content, hence corrosivity, of deep waters, reflecting either

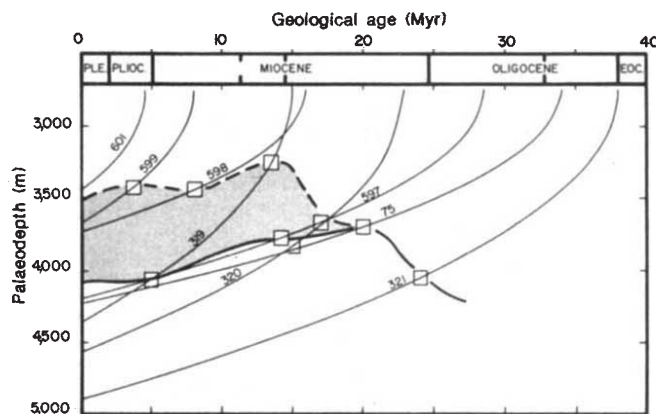


Fig. 2 CaCO_3 accumulation record plotted on age–depth curves for each site. Squares are 1 Myr $\times$ 100 m and provide an indication of accuracy. The shaded region indicates the depth zone of the lysocline. Solid line, CCD; dashed line, lysocline.

stagnation or enhanced input of organic carbon, and (2) reduced input of biogenic CaCO_3 to the deep ocean. The ultimate causes of changes in these parameters have been related to: the rate of formation of oceanic bottom water; the nature of shelf/basin depositional fractionation which depends largely on sea level; and sea-surface biological productivity of calcium carbonate and organic carbon. Oxygen isotope data suggest that the middle Cenozoic is characterized by constant or perhaps even declining ice volume^{24,25}, hence only minor changes in the production rate of bottom water may have occurred then. Sea level rose gradually from the mid-Oligocene to the mid-Miocene²⁶, consistent with a shoaling CCD, but as the sudden, large mid-Oligocene sea-level fall does not affect the CCD, it seems inappropriate to invoke the ensuing gradual rise of sea level. We therefore agree with the view^{15,22,23} that, during times of constant ice volume, sea-surface biological productivity exerts the dominant control of the CCD. The problem remains complex, however, because factors such as total carbonate supply to the sea floor¹⁵, the amount of non-calcareous, siliceous productivity²⁷, and how or why productivity changes during times of relatively uniform oceanic and atmospheric circulation, are not well understood on a global scale.

Van Andel *et al.*¹⁵ have suggested that the lysocline may be related to the volume of corrosive deep water. If this is the case, our data suggest an increase in this volume which, within the accuracy of our data, occurred during the same time span as the inferred ice build-up on Antarctica, 16.0–12.4 Myr (oxygen-isotope stratigraphy from ref. 28; foraminifer zonation and timescale from ref. 29). We note, however, that the present lysocline does not correspond to any water mass boundaries in the Pacific. More recently, Broecker and Peng³⁰ have suggested that the thickness of the zone of rapid CaCO_3 dissolution between the lysocline and the CCD varies directly with the downward flux of calcite. If so, the early Miocene separation of the lysocline and CCD may be related to increased surface-water productivity. There exists ancillary evidence supporting increased equatorial productivity of both calcareous¹⁵ and siliceous²⁷ organisms then.

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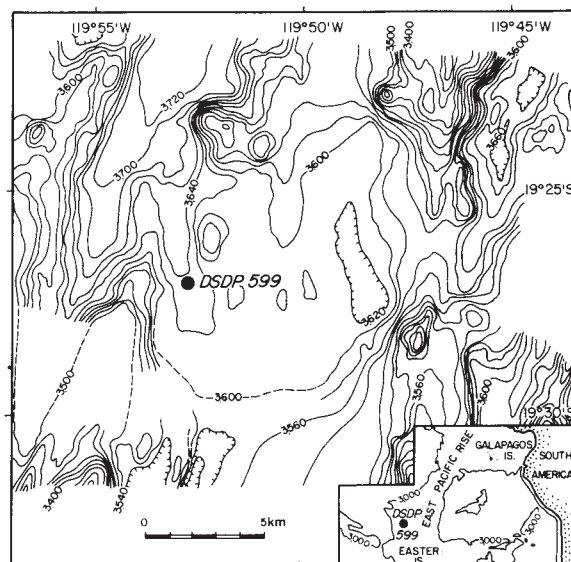


Fig. 1 Bathymetry (from seabeam swath-mapping⁴) and location of Deep Sea Drilling Site 599 on the west flank of the East Pacific Rise. This site, along with the other DSDP Leg 92 sites, was the first on the East Pacific Rise to be drilled using the hydraulic piston corer, which minimizes sediment disturbance during the coring process.

Downslope transport of metalliferous sediments along the East Pacific Rise during the late Miocene

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The distribution of metalliferous sediments next to active spreading centres has scientific and economic interest¹. Although metal-rich waters emanating from active hydrothermal vents have been traced in intermediate level water masses far beyond the ridge crest², the greatest concentrations of metal oxides in sediments occur near the vents³. There, however, it is conceivable that the oxides may be redistributed and possibly further concentrated by redeposition, resulting in misconceptions of the age and relative timing of hydrothermal pulses. Here, we document microfossil evidence of stratigraphical inversion and redeposition of Upper Miocene (Messinian) sediments cored at Deep Sea Drilling Project (DSDP) Site 599 on the East Pacific Rise (EPR). This suggests that where reworking can be confirmed, care should be taken not to correlate directly the occurrences of the metalliferous sediments with apparent coeval pulses of hydrothermal activity or enhanced sea-floor spreading rates.

Hole 599 (19°27.09' S, 119°52.88' W; water depth = 3,654 m), located ~600 km west of the present ridge crest, was drilled in a small basin surrounded by a region of low relief with abyssal hills ranging from 200 to 300 m in height (Fig. 1). This site yielded some 41 m of mostly Upper Miocene clay-bearing to clayey calcareous oozes with a basement age of 8.1-8.6 Myr (ref. 5). The lower 31 m of the section is strikingly layered, consisting of alternating light (mostly yellowish brown to dark yellowish brown) and dark (mostly dark reddish brown) coloured layers tens of centimetres thick (Figs 2, 3). The colour variations are the result of changes in the relative amounts of calcium carbonate (70-80% in light coloured sediments, 55-70% in dark coloured sediments⁵) and a noncarbonate fraction which is primarily clays and ferruginous grains. The clays are mixtures of poorly crystalline smectites and amorphous oxides. The ferruginous grains, which range in size from 10 to 100 µm, are a metalliferous hydrothermal component which has been described by Leg 34 scientists as red-brown to yellow-brown, semi-opaque oxides (RSOs)⁶.

The strongly layered portion of the section is dated between

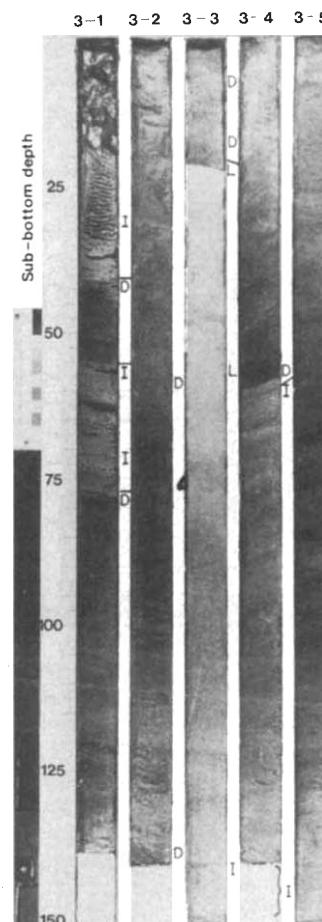


Fig. 2 Sections 1-5 of DSDP Core 599-3 showing alternations of light (L), dark (D), and intermediate (I) coloured lithologies within the Messinian clayey nannofossil ooze sequence (see key in Fig. 3 for detailed colour descriptions). The darker lithologies contain less carbonate and more hydrothermal metalliferous components than do the lighter lithologies. Note the sharp contact (dark over light) at 21 cm in section 599-3-3.

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'Strangelove ocean' before the Cambrian explosion

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The Palaeozoic and Mesozoic eras were terminated by faunal changes involving mass extinction of the old and explosive evolution of the new fauna, but the fossil record shows only a Cambrian Explosion at the end of the Precambrian. Stanley speculated that the explosion was only possible after the ubiquitous algae community had been largely eliminated¹; ecological niches were thus liberated for explosive evolution. If the Cambrian Explosion were preceded by a mass mortality (or by a mass extinction), such an event should leave a record in the form of geochemical anomalies. We have undertaken a search for geochemical anomalies at the Precambrian/Cambrian contact. We report here the discovery of a sharp negative carbon-isotope shift in the carbonate of a clay immediately above a marker in the Precambrian/Cambrian boundary, the China C marker, and interpret this signal as evidence of sudden decrease in fertility before the Cambrian explosion of invertebrate evolution. The discovery suggests that the Precambrian/Cambrian boundary might be defined by an event-marker at a palaeontologically correlative horizon.

Investigations of the Cretaceous/Tertiary boundary reveal that the boundary event left a signature in an iridium anomaly and a carbon isotope anomaly^{2,3}. The dual Ir- $\delta^{13}\text{C}$ anomalies were also found recently in a Permian/Triassic boundary clay⁴. The exact placement of Precambrian/Cambrian boundary has not yet been established. Chinese scientists have suggested three datum levels as candidates: China A marks the first appearance of inarticulate brachiopods and China B defines the base of the *Paragloborilus Siphononuchites* Zone, characterized by a more diversified shelly fauna. The third, or the China C marker, is slightly above the China B (Fig. 1), and defined by a remarkable lithological change from a phosphate-rich rock to a black shale. This horizon marks the base of a formation containing the first trilobites⁵, and has been correlated for thousands of kilometres across South China.

Following the discovery in 1982 of an iridium anomaly in a black shale above the China C marker (C. C. Woo, personal communication) one of us (K.J.H.) took samples across the iridium-rich horizon. Six samples from a 12-m section were obtained at the Type Locality for the Precambrian Sinian Group in the Yangtze Gorge section, ~20 km west of Ichang, Hupeh Province, China. Analysis confirmed the presence of higher

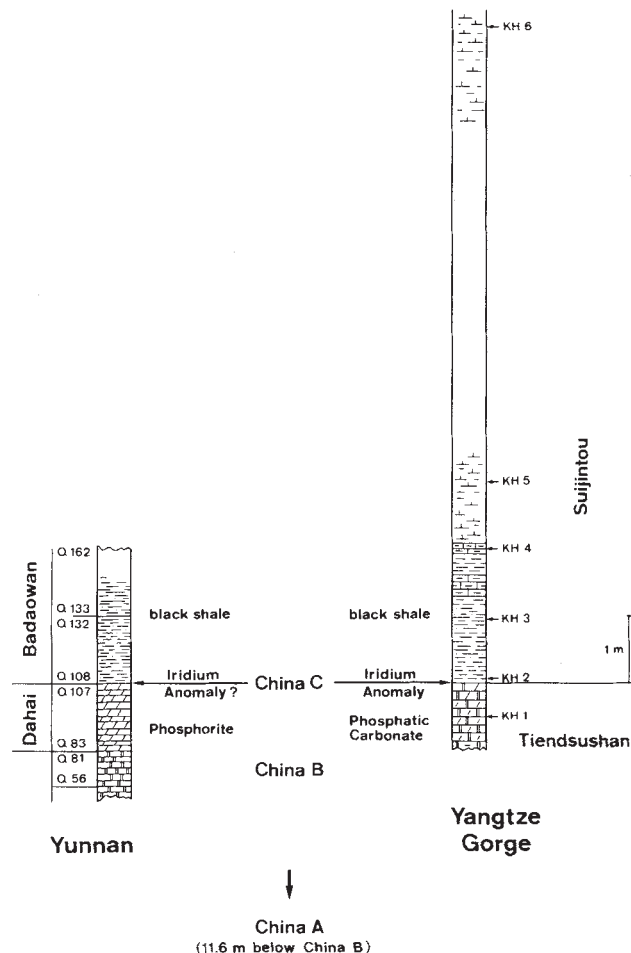


Fig. 1 Stratigraphical columns across a proposed Precambrian/Cambrian contact (China C marker) at two localities in south-west China. Iridium anomaly has been identified in the boundary clay at the Yangtze Gorge locality. The Q and KH numbers are sampling numbers. The Q samples have been obtained at 2-cm intervals apart. KH samples were taken for a reconnaissance study. Not shown are the densely spaced samples from the Yangtze locality. China B marks the base of Dahai.

iridium and osmium contents in a black clay immediately above the China C marker. Also found was a carbon-isotope anomaly at the iridium-enriched horizon (Fig. 2).

The discovery prompted us to resample across the China C marker in 1984 to produce a more systematic investigation. Fifty-three samples were taken from the Yangtze Gorge locality near the marker at intervals spaced 2-cm apart. Some 100 samples were taken from the Kunyang Mine with 2-cm sampling intervals, not only across China C, but also across China A and China B markers. The close sampling was done to define precisely the carbonate-isotope anomaly, and to determine whether the anomaly is a signal or a random variation. The stratigraphy of both localities has been described in detail elsewhere^{5,6}. Note that the first trilobite specimens were collected in a calcareous shale within 1 m above the China C marker at the Yangtze locality, but at a horizon some 10 m higher at Kunyang.

The isotope analyses were carried out at ETH Zurich. The phosphatic rocks contain 90% carbonate and the black shale contain between 8 and 26% carbonate, which is mainly dolomite. A thin seam of black shale immediately above the China C marker can, however, be considered a boundary clay layer; the sample at the Kunyang Mine contains 0-4% carbonate, and the one at the Yangtze Gorge locality is practically carbonate-free, thus yielding no isotope results.

The $\delta^{13}\text{C}$ values of the dolomite at the two localities are shown by Fig. 3. The background values are slightly more negative at

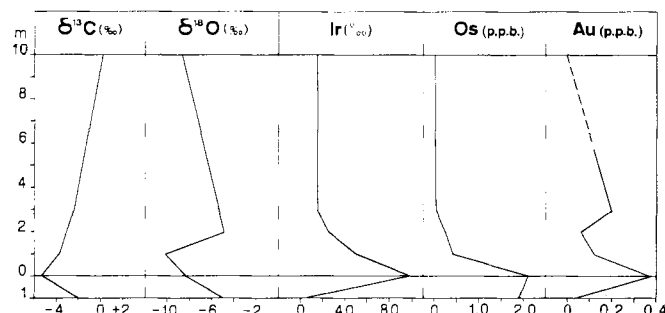


Fig. 2 Geochemical anomalies across the China C marker at Yangtze locality.

the Yangtze Gorge than those at the Kunyang locality. Note the sharp anomaly of $\sim 5\%$ more negative values exactly at a horizon immediately above the China C marker. This anomaly is present at both localities, which are $\sim 1,000$ km apart.

The sedimentation rate of the Cambrian black shale cannot be accurately determined. Chinese scientists estimated an average sedimentation rate of <10 m Myr^{-1} , for the Cambrian rocks, based on the thickness of the Cambrian strata ($<1,000$ m) of the Yangtze Gorge Section⁶ and the duration of the Cambrian Period (>100 Myr). Assuming such a rate, the isotope shift at the China C datum should have taken place within 2,000 yr. The anomaly is thus a sharp perturbation, comparable in its magnitude and sharpness with those found across the Cretaceous/Tertiary and Permian/Triassic boundaries.

Samples across the China A and China B markers at Kunyang are still being analysed. We have not yet found iridium nor carbon-isotope anomalies across those boundaries.

The values of $\delta^{13}\text{C}$ in marine carbonate is influenced by many factors, such as terrestrial influx, ocean-atmosphere exchange, and biological productivity⁷. Thousands of analyses of deep-sea drill cores indicate that carbonate-isotope values of ocean sediments do not vary rapidly with time, on account of the very large reservoir of dissolved carbonate atoms in ocean waters. No sharp global anomalies ($2\text{--}4\%$ in 10^3 or 10^4 yr) have ever been observed except across the Cretaceous/Tertiary boundary, although long-term variations with anomaly values up to 4% over a $10^6\text{--}10^7$ -yr period are not uncommon.

Sharp perturbation of ^{13}C values in sediments has been related to ocean fertility. Natural fractionation of carbon isotopes by biological activity is well known. Carbon-12 atoms are preferentially used by living organisms, so that the carbon-13 atoms in dissolved carbonates are enriched in surface waters where the planktons flourish. This phenomenon has been confirmed by isotope analyses of dissolved carbonates in ocean and lake waters^{7,8}. Sudden changes in biological productivity produce $\delta^{13}\text{C}$ anomalies in surface water. Two types of anomalies have been recognized, namely a positive anomaly of 'fertility perturbation' and a negative anomaly or 'Strangelove perturbation'.

The 'fertility perturbation' was discovered by McKenzie at Lake Greifen⁹. There was a positive anomaly of 4.5% in the inorganic carbon of the surface waters during late July and August (months of high fertility, when green algae production reaches a maximum) (Fig. 2). No such anomaly was detected in the inorganic carbon of the bottom sediments. In fact, a slight negative anomaly of $<1\%$ was detected in September, the month after the harvest, when the light carbon in bottom waters was relatively enriched because of the decay of dead organisms.

The exact opposite type of perturbation has been found at Cretaceous/Tertiary and Permian/Triassic boundaries^{3,4}. In ancient as in modern ocean waters, the inorganic carbon of surface waters is normally enriched in carbon-13. The $\delta^{13}\text{C}$ is $\sim 2\%$, more positive than that of bottom waters because of the steady production of plankton. Fertility is a normal state. After events such as those marking era-boundaries, the environmental pollution may have been such as to reduce or to suppress

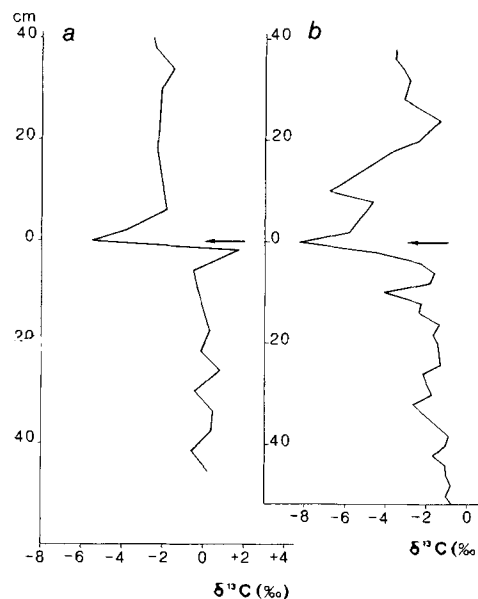


Fig. 3 Strangelove perturbation across the China C marker. *a*, Kunyang Mine locality, Yunnan Province. *b*, Yangtze Gorge locality.

plankton production for thousands of years. This hypothetical state of ocean, almost devoid of living organisms, has been called the 'Strangelove Ocean' (refs 8, 9). The geochemical expression of this ocean is a perturbation in the $\delta^{13}\text{C}$ value of planktonic sediments: when the carbon-isotope fractionation by biological activity ceases, a mixing of surface and bottom waters should produce a carbon-isotope composition that is consistent from the top to the bottom of the water column. Consequently, the planktonic sediments deposited during a crisis should have a $\delta^{13}\text{C}$ value similar to that in the bottom sediments, and be considerably more negative than the planktonic sediments deposited in normally fertile conditions. The presence of a Strangelove perturbation across the Cretaceous/Tertiary boundary is thus considered to be evidence of an interrupted plankton production following a boundary event⁹.

Calcareous planktons, such as foraminifera and nannoplanktons, had not yet evolved during the Precambrian. Yet Precam-

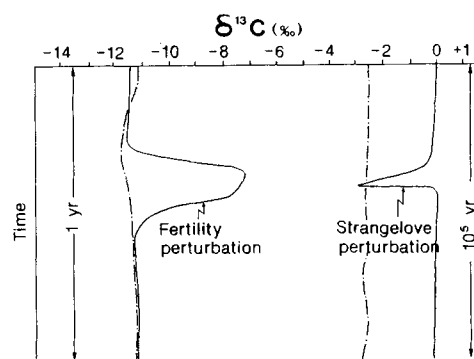


Fig. 4 Carbon-isotope anomaly related to plankton productivity. The left-hand side shows the annual variation of carbon-isotope values in dissolved carbonates of surface water (solid line) and of bottom water (dashed line) in Lake of Greifen, Switzerland. Time at the bottom is January and at the top is December. A positive perturbation in surface water is observed in July and August when the plankton fertility reaches the maximum. The right-hand side of the figure is the short-term perturbation of carbon-isotope values across the Cretaceous/Tertiary boundary at DSDP Site 524 in the South Atlantic Ocean, showing the changes in the planktonic (solid line) and in the benthic (dashed line) foraminifers. The base of the negative Strangelove perturbation coincides with the Cretaceous/Tertiary boundary, marked by an iridium anomaly; and the perturbation is believed to indicate that the biomass of ocean planktons had been drastically reduced.

brian pelagic or hemipelagic sediments contain carbonates. The carbonates were probably produced through the biological activity of planktonic organisms, such as phytoplanktons or green algae. These left no fossil skeletons, but their photosynthesis removed CO₂ from the surface water to cause carbonate oversaturation and precipitation. This process of calcite production occurs annually in the Swiss lakes¹⁰. If a catastrophe should have occurred, and resulted in suppressed plankton production in Precambrian oceans, the biological fractionation of carbon isotopes would cease. Consequently, we could interpret the large negative $\delta^{13}\text{C}$ anomaly at the 'China C datum' as a Strangelove perturbation. Geochemical anomalies thus support the theory¹ that early Cambrian evolution of multicellular life was preceded by a catastrophic destruction of the late Precambrian biomass.

Some unknown single-celled herbivores were held responsible for the destruction of the ubiquitous algal community of the Precambrian¹. The idea is not supported by palaeontological evidence, because the postulated culprit left no fossil skeletons. Furthermore, the Cambrian explosion was preceded by the evolution and extinction of the Ediacarian soft-bodied fauna¹², which could not have been vulnerable to the herbivores in ref. 1. If there was a major extinction before the Cambrian explosion, some other cause must be sought.

Note that an iridium anomaly in the black shale immediately above the China C marker has been found in many parts of South China^{12,13}. The anomalously high values up to 30 p.p.b. (parts per 10⁹) Ir (ref. 12) in metalliferous sediments may have resulted from secondary enrichment, but the enrichment may have been preceded by a primary source of iridium. The possibil-

ity of a large-body impact before the Cambrian explosion must thus be considered an attractive working hypothesis.

The dual iridium $\delta^{13}\text{C}$ anomaly in a boundary clay above a palaeontological datum has only been found globally across the Cretaceous/Tertiary boundary and, in China, across the Permian/Triassic boundary. The presence of the same association of an iridium-anomaly/Strangelove-perturbation/boundary-clay above a palaeontological datum marking the first appearance of trilobites cannot be coincidental. Whatever the ultimate cause of the geochemical anomalies, the possibility that the Precambrian/Cambrian boundary is definable by an event marker is worthy of further exploration. The IGCP Project 199 on Rare Events in Geology has undertaken the task of international cooperation to ascertain if geochemical anomalies similar to those reported here can be found elsewhere.

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Mass extinction among non-marine tetrapods

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The fossil record of non-marine tetrapods (amphibians, reptiles, birds and mammals) has been described by numerous authors¹⁻³, and major ecological replacements, mass extinctions and adaptive radiations have been identified. However, most of these features of the large-scale evolution of tetrapods have been noted without numerical data of the kind assembled for marine invertebrates⁴⁻¹⁰, marine vertebrates⁷⁻¹⁰ and vascular land plants¹¹. Much has been learnt from the record of marine invertebrates, particularly about the overall patterns of diversification with time, the performance of different major taxonomic groups at different times, and the magnitude and timing of mass extinctions. The present study tests some of the general conclusions on the basis of a new compilation of data on the fossil record of terrestrial tetrapods. I show that family diversity rose with time, and in particular from the Cretaceous to the present day. There were several mass extinction events, but none of these was associated with a statistically high extinction rate. The extinction events, including the famous terminal Cretaceous extinction, were the result of a slightly elevated extinction rate combined with a depressed origination rate, and the present evidence does not support the view that mass extinctions are statistically distinguishable from background extinctions. Further, the record of non-marine tetrapods shows an increasing total extinction rate and an only marginally decreasing probability of extinction (per-taxon rate) from the late Devonian to the present, the opposite of the findings from the record of marine animals.

The present study is concerned with the fossil record of non-marine tetrapods: this includes terrestrial, freshwater and flying forms, but excludes fully marine families. A list of all the families of terrestrial tetrapods was made using the most recent taxonomic reviews, as well as secondary literature, to the end of 1984 (for details see ref. 12). A small number of extinct

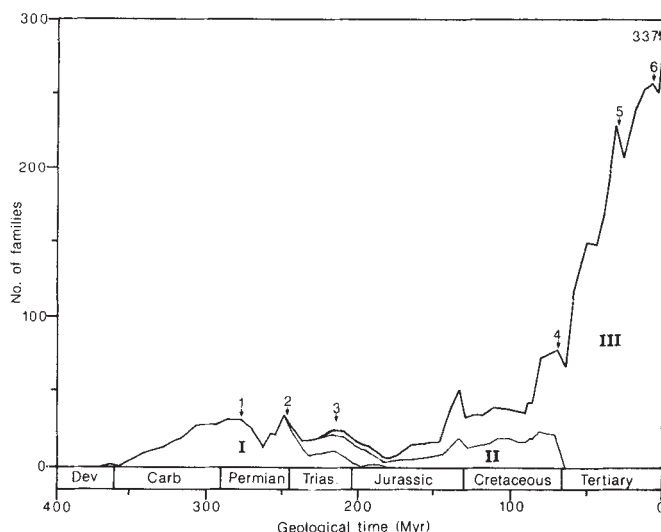


Fig. 1 Standing diversity with time for families of terrestrial tetrapods. The upper curve shows total diversity with time, and six apparent mass extinctions are indicated by drops in diversity, numbered 1-6. The relative magnitude of each drop is given in terms of the percentage of families that disappeared: (1) early Permian (Sakmarian-Artinskian), 58%; (2) late Permian-early Triassic (Tatarian-Scythian), 49%; (3) late Triassic (Norian-Rhaetian), 28% (4) late Cretaceous (Maastrichtian), 14%; (5) early Oligocene (Rupelian), 8%; late Miocene (Tortonian-Messinian), 2%. The mass extinctions were produced by a combination of a slightly elevated extinction rate and a reduced origination rate. The timescale used was that of Palmer²⁶. Three assemblages of families succeeded each other through geological time: I, labyrinthodont amphibians, anapsids, mammal-like reptiles; II, early diapsids, dinosaurs, pterosaurs; III, the 'modern' groups: frogs, salamanders, lizards, snakes, turtles, crocodiles, birds, mammals. These assemblages were defined somewhat arbitrarily to encompass those groups that dominated non-marine tetrapod faunas in the late Palaeozoic, the Mesozoic and the Cenozoic respectively: the data are not robust enough for the application of the factor analytic technique of Sepkoski⁶. Abbreviations: Carb., Carboniferous; Dev., Devonian; Trias., Triassic.

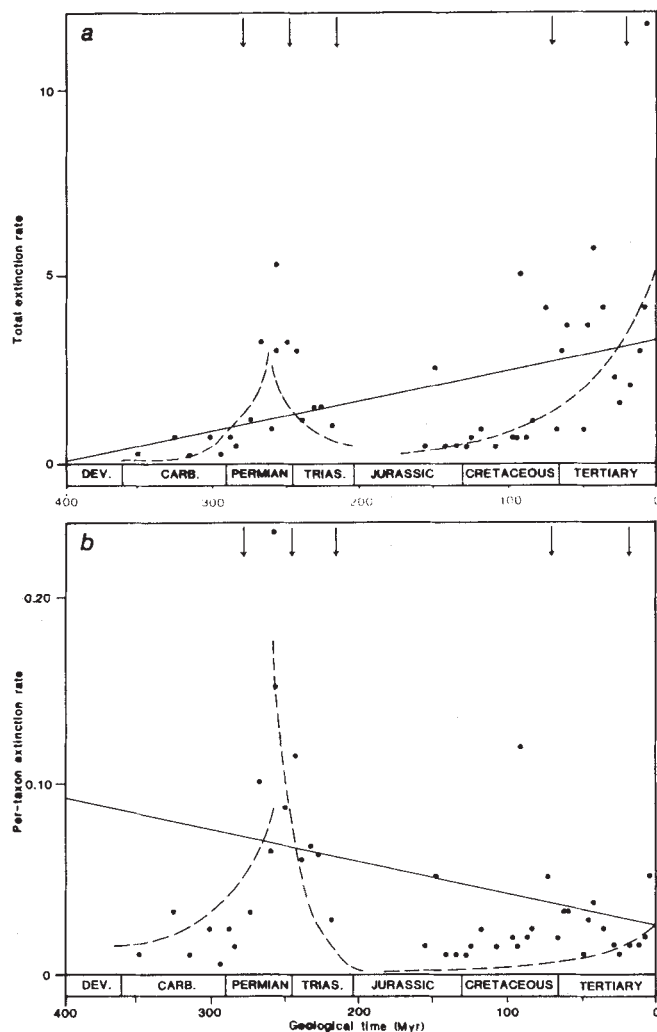


Fig. 2 Total extinction rate (*a*) and per-taxon extinction rate (probability of extinction) (*b*) for families of terrestrial tetrapods. The total extinction rate increases with time according to the linear regression $y = 0.00832x + 3.497$ ($P = 0.003$; $r^2 = 0.16$), while the per-taxon extinction rate declines with time according to the linear regression $y = -0.00013x + 0.017$ ($P = 0.024$; $r^2 = 0.09$). The extinction rate data in both cases are better explained by three log linear regressions for different time divisions (these are indicated by dashed curves; see Table 1 for the equations). Extinction rates were calculated for 45 stages between the early Carboniferous and the Pleistocene: the late Devonian (Famennian) and early to middle Jurassic stages (Hettangian–Oxfordian) were excluded because of the poor fossil record within these intervals. The episodes of mass extinction determined in Fig. 1 are indicated by arrows. Abbreviations as in Fig. 1.

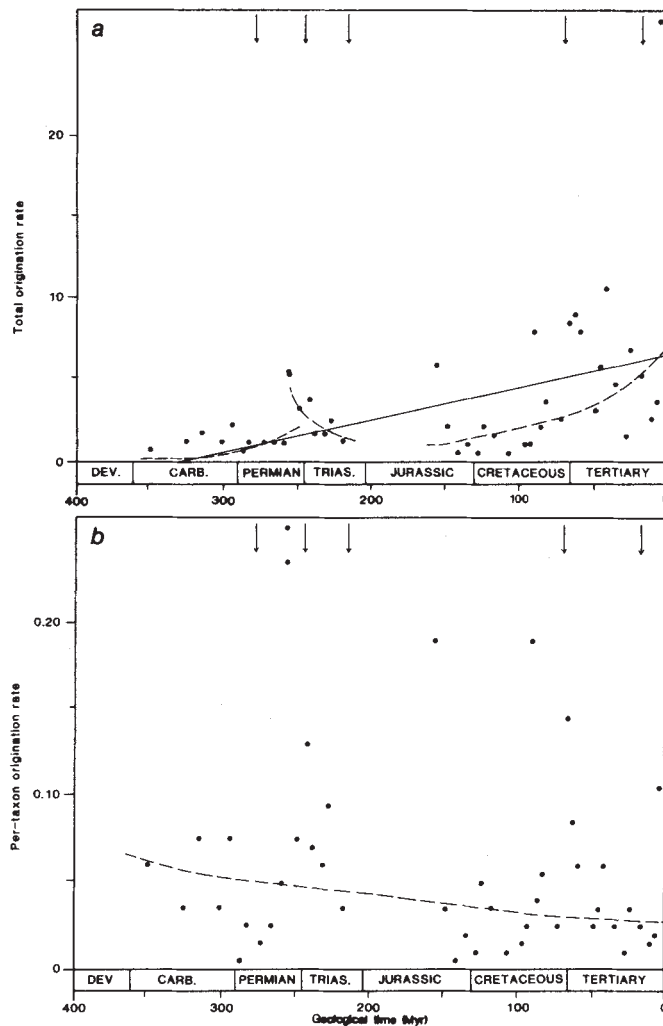


Fig. 3 Total origination rate (*a*) and per-taxon origination rate (probability of origination) (*b*) for families of terrestrial tetrapods. The total origination rate increases with time according to the linear regression $y = 0.02008x + 6.356$ ($P = 0.001$, $r^2 = 0.21$), while the per-taxon rate declines with time according to the log linear regression, $\log y = 0.00105x - 1.582$ ($P = 0.048$, $r^2 = 0.07$). The total origination rate data are better explained by three log linear regressions for different time divisions (these are indicated by dashed curves; see Table 2 for equations). The per-taxon origination rate data did not correspond to a statistically significant linear regression, nor were significant regressions derived for different time divisions: only the late Permian to late Triassic data fell along a significant linear or log linear regression (for the equations, see Table 2). Other information is given in Fig. 2 legend.

families were excluded from the analysis as they were based on single, often incomplete, specimens; this left a total of 730 families, of which 469 are extinct. The quality of the fossil record of terrestrial tetrapods is variable because certain kinds of environments are poorly preserved, and there is evidence that the record is particularly bad in the early and middle Jurassic, and in the mid-Cretaceous (see below). The range in geological time for each family was determined from the most recent available literature, and this was resolved to the level of the stratigraphic stage (duration 2–19 Myr; mean duration 6 Myr).

The numbers of families of terrestrial tetrapods are plotted against geological time in Fig. 1. The most striking feature of this graph is that the number of families present at any time remained at levels below about 50 until the last quarter of the Cretaceous period when the numbers of families increased to nearly 80, and the rise continued to the present total of 337 in several steps during the Tertiary. This great increase in family diversity may be partly an artefact of the fossil record which

generally improves towards the present, and of the greater research emphasis devoted to Tertiary fossil vertebrates than to earlier ones. However, a large part of the increase is probably a real biological phenomenon, as has been shown for the record of marine invertebrates^{13,14}.

The second interesting feature of this analysis is that it shows a succession of family assemblages that dominated for a time and were then replaced (Fig. 1). The first assemblage of tetrapod families—labyrinthodont amphibians, anapsids and mammal-like reptiles—dominated from the late Devonian to the early Triassic. The second assemblage—primitive diapsid reptiles, thecodontians, dinosaurs and pterosaurs—arose in the Permian and increased in diversity through the Triassic as the members of the first assemblage declined. This second assemblage dominated from the late Triassic to the late Cretaceous. The third assemblage, which includes all modern tetrapod groups—frogs, salamanders, lizards, snakes, turtles, crocodiles, birds and mammals—arose in the late Triassic, and increased in diversity

through the Jurassic and early Cretaceous. During the Cretaceous, the diversity of these modern groups was approximately equal to that of the second assemblage of dinosaurs and pterosaurs. In the latest Cretaceous, the diversity of the modern groups rose dramatically and became twice that of the dinosaurs and pterosaurs, long before the terminal Cretaceous extinction event. The modern groups were barely affected by this extinction, and their family diversity increased through the Tertiary. Members of the first assemblage reached an observed familial diversity of about 30 in the Permian, whereas dinosaur and pterosaur families reached an observed diversity of only 20–25 families in the Cretaceous.

The third part of this study concerns the occurrence of mass extinctions in the fossil record of terrestrial tetrapods. The terminal Cretaceous event, during which the dinosaurs and pterosaurs disappeared, is well known^{15–17}, and other extinctions among tetrapods have been suggested in the middle of the Permian^{18,19}, at the end of the Permian^{18,19}, at the end of the early Triassic^{18,19}, and at the end of the Triassic^{18–20}. A mass extinction may be identified at first by the observation of a drop in diversity. The general upward trend of the plot of family diversity of terrestrial tetrapods with time (Fig. 1) is interrupted by several major reductions in family diversity, in the early to mid-Permian (Sakmarian–Artinskian), the late Permian to early Triassic (Tatarian–Scythian), the late Triassic to early Jurassic (Norian–Hettangian), the late Jurassic (Portlandian), the late Cretaceous (Maastrichtian), the early Oligocene (Rupelian) and the late Miocene (Tortonian–Messinian).

These episodes of diversity decrease may not all represent mass extinctions. Two of them (early Jurassic, late Jurassic) are followed by times during which there is a very poor fossil record of terrestrial tetrapods (that is, there are no fossils known, or only one or two, from a particular stratigraphic stage). In these two cases, much of the decline in diversity, if not all of it, could be the result of the poor fossil record, and the conservative conclusion is to assume that these two are not mass extinctions. The initial part of the late Triassic to early Jurassic mass extinction began well before the poor fossil record of the early to middle Jurassic, and the earlier part is retained as an extinction event¹². The mid-Permian decline in diversity might also partially reflect a gap in the fossil record, but, again, at least the earlier part is probably a mass extinction.

The six remaining declines in diversity are listed in Fig. 1 as a percentage of the total number of families present at the start. These vary in magnitude from 2% in the late Miocene to 58% at the end of the early Permian. The first three extinctions, in the Permian and Triassic, had the greatest overall effects (falls of 28–58%), but each of these lasted over two stratigraphic stages (durations of 14, 13 and 17 Myr for extinctions 1–3 respectively). The terminal Cretaceous event had a lower overall effect (drop of 14%), but it occurred in a single stratigraphic stage (duration 8 Myr). The two Tertiary extinctions (numbers 5 and 6) had much smaller relative effects on overall diversity.

A decline in family diversity could be the result of a high extinction rate, or a low origination rate, or a combination of the two^{18,21}. It is commonly assumed that declines in diversity (mass extinctions) are caused only by a high extinction rate, and reasons are then sought to explain the 'dying off'. However, if the decline in diversity were associated with a depressed family origination rate, and the extinction rate was normal, we would have to seek other kinds of explanations.

The nature of the six terrestrial tetrapod mass extinctions was explored by an analysis of the total extinction and origination rates (simply the numbers of families dying out or appearing during a stage, divided by the duration of that stage), and the probabilities of extinction or origination (calculated for each stage as the total rate divided by the number of taxa 'at risk' or 'available', according to Van Valen's²² definition; also called per-taxon rates).

First, the total and per-taxon rates were plotted against geological time (Fig. 2) to determine whether the times of observed mass extinctions were marked by high extinction rates. Most

Table 1 Regression equations for total rates and per-taxon rates (probabilities) of extinction for non-marine tetrapods from the late Devonian to the present

	<i>P</i>	<i>r</i> ²
a Total rates		
(1) (All stages) $y = 0.00832x + 3.194$	0.003	0.16
(2) (All stages) $\sqrt{y} = 0.00301x + 1.650$	0.001	0.20
(3a) (Kimm–Pleist) $\log y = 0.00841x + 0.698$	0.000	0.53
(3b) (Kaz–Nor) $y = -0.06644x - 13.903$	0.007	0.73
(3c) (Kaz–Nor) $\log_{10} y = -0.01652x - 3.709$	0.005	0.76
(3d) (Tour–Ufim) $\log_{10} y = 0.01872x + 5.153$	0.001	0.75
b Per-taxon rates (probabilities)		
(4) (All stages) $y = -0.00013x + 0.017$	0.024	0.09
(5a) (Kimm–Pleist) $\log_{10} y = 0.00138x - 1.729$	0.182	0.03*
(5b) (Kaz–Nor) $y = -0.00275x - 0.573$	0.005	0.76
(5c) (Kaz–Nor) $\log_{10} y = -0.01666x - 5.099$	0.002	0.84
(5d) (Tour–Ufim) $\log_{10} y = 0.01247x + 2.054$	0.006	0.56

Probabilities of extinction were calculated using the Van Valen²² metric (that is, the number of extinctions (last appearances) in a stage, divided by the number of taxa at risk. The latter was estimated as the number of taxa at the beginning of the interval, minus half the extinctions, plus half the originations in the interval). The equations that give the highest percentage of explained sum of squares (*r*²) are listed here. Equations are given for all stages treated together, and then for a three-way division of the data, with splits in the mid-Permian (Ufim/Kaz) and in the early to middle Jurassic (Nor/Kimm). Note the absence of data for the stages of the early and middle Jurassic. The probability (*P*) of the correctness of each regression equation is also given; Kaz, Kazanian; Kimm, Kimmeridgian; Nor, Norian; Pleist, Pleistocene; Tour, Tournaisian; Ufim, Ufimian.

* *P* > 0.1 (nonsignificant results) are marked by an asterisk.

total extinction rates fell below 4 per Myr, but several rates were higher (up to 12 family extinctions per Myr), while most probabilities of extinction fell below 0.120 per family per Myr, with higher rates up to 0.240. Both graphs gave highly significant linear regressions (*P* = 0.003, *P* = 0.024; Table 1). Raup and Sepkoski⁷ suggested that mass extinction events were associated with statistically very high extinction rates (outliers above the 95% confidence envelope of a linear regression of all extinction rates), but this technique did not work for the non-marine tetrapod record. Most of the outliers (that is, stages during which extinction rates were statistically very high, for example, Pleistocene, Ufimian), did not correspond to falls in overall diversity, as they were matched by even higher origination rates. The technique also did not work when the extinction data were transformed to logarithms²³ or to square roots²⁴.

The total and per-taxon origination rates were then plotted against geological time (Fig. 3) to determine whether the times of observed mass extinctions were marked by statistically low origination rates. The graph of total origination rates (Fig. 3a) gave a highly significant linear regression (*P* = 0.001), but the graph of probabilities (Fig. 3b) did not (Table 2). Statistical tests for outliers—extremely low origination rates—did not pick out the times of mass extinction. The statistically low rates did not correspond to falls in diversity because they were matched by high extinction rates.

Thus there is no evidence in the record of non-marine tetrapods that mass extinctions are statistically distinguishable from 'background' extinctions, by having either a very high family extinction rate or a very low family origination rate. Note, however, that the time resolution in this study, as in most others

Table 2 Regression equations for total rates and per-taxon rates (probabilities) of origination for non-marine tetrapods from the late Devonian to the present

a Total rates	P	r ²
(1) (All stages) $y = 0.02008x + 6.356$	0.001	0.21
(2) (All stages) $\log_{10} y = 0.00254x + 0.636$	0.000	0.28
(3a) (Kimm-Pleist) $\sqrt{y} = 0.01333x + 2.790$	0.001	0.31
(3b) (Kimm-Pleist) $\log_{10} y = 0.00629x + 0.849$	0.002	0.29
(3c) (Kaz-Nor) $\log_{10} y = 0.01804x - 4.008$	0.009	0.71
(3d) (Tour-Ufim) $\log_{10} y = 0.00580x + 1.556$	0.085	0.22
b Per-taxon rates (probabilities)		
(4) (All stages) $y = -0.00010x + 0.042$	0.137	0.03*
(5) (All stages) $\log_{10} y = -0.00105x - 1.582$	0.048	0.07
(6a) (Kimm-Pleist) $y = -0.00013x + 0.038$	0.286	0.01*
(6b) (Kimm-Pleist) $\log_{10} y = -0.00084x - 1.584$	0.337	0.01*
(6c) (Kaz-Nor) $y = -0.00425x - 0.906$	0.035	0.51
(6d) (Kaz-Nor) $\log_{10} y = -0.01816x - 5.394$	0.017	0.63
(6e) (Tour-Ufim) $y = 0.00052x + 0.204$	0.247	0.05*
(6f) (Tour-Ufim) $\log_{10} y = -0.00038x - 1.517$	0.468	0.00*

Probabilities of origination were calculated using an analogue of Van Valen's²² extinction probability metric. This, and other conventions and abbreviations, are explained in the legend to Table 1.

published so far, is very coarse, and this could minimize the true size of a sudden event by averaging it out over several million years. In all cases, the declines in diversity (mass extinctions) were produced by slightly elevated extinction rates and slightly depressed origination rates. However, neither the total rates nor the probabilities were obviously different from 'normal' rates, and this statistical study points to a model of extinction events that does not distinguish mass extinctions as a discrete class of phenomena from background extinctions.

The graph of total extinction rates (Fig. 2a) shows that there was an overall rising trend with time. Most of this rise is explained by the increase in overall diversity (the more families there were, the more could become extinct in a unit of time). However, the probability of extinction (Fig. 2b) decreased with time, although by only a very small amount (0.00012 less families dying out per family per Myr; ~0.07% decline per stage). The increase in total extinction rates, and the small decrease in probability of extinction are very surprising, as the record of marine animals shows an overall markedly declining trend in both total and per-taxon extinction rates, the latter trend giving a decline of ~6.6% per stage^{7,22,25}. The graph of total origination rate (Fig. 3a) shows a rising trend with time, while the graph of probability of origination (Fig. 3b) did not yield a significant regression.

An attempt was made to find statistical models that described the data for extinction and origination rates more precisely (that is, giving a higher percentage of explained sum of squares, r^2), by splitting the data into several time intervals, and by transforming the rates into logarithms and square roots. The data set was not large enough, after refinement, for time series analysis^{10,25}, but it was found that the extinction rate data could be better described if the data were divided into three time sets: from the early Carboniferous (Tournaisian) to the mid-Permian (Ufimian), from the late Permian (Kazanian) to the late Triassic (Norian), and from the late Jurassic (Kimmeridgian) to the

Pleistocene. A set of three log linear regressions gave the best explanation of the total extinction rate data (Table 1, equations 3a-d), and the same applied to the per-taxon extinction rate data, although the third time interval was still poorly explained (Table 1, equations 5a-d). For total origination rates, three log linear regressions also provided the best model (Table 2, equations 3a-d), but the per-taxon origination rate data could not be so readily accounted for (Table 2, equations 6a-f). The best-fit regressions divide the data into three time segments (Figs 2, 3): (1) early Carboniferous to mid-Permian (rates rise sharply); (2) late Permian to late Triassic (rates fall sharply), (3) late Jurassic to Pleistocene (rates rise again).

The present analysis of the fossil record of non-marine tetrapods has shown two main points. First, none of the observed mass extinctions was caused by either very high rates of extinction or very low rates of origination. In fact, each event was associated with rates that did not differ statistically from normal background rates. Second, the total rate of extinction has risen with time from the late Devonian to the present day, and the probability of extinction has declined only marginally. The tetrapod record shows little evidence for overall improvements in the ability of organisms to 'resist' extinction—increases in darwinian fitness²².

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Susceptibility to murine lymphocytic choriomeningitis maps to class I MHC genes—a model for MHC/disease associations

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Susceptibility to some human diseases is linked, albeit weakly, to major transplantation antigens (HLA) encoded by the major histocompatibility gene complex (MHC)^{1,2}. Here we have studied MHC/disease association in inbred strains of mice after intracerebral (i.c.) injection of lymphocytic choriomeningitis virus (LCMV)³⁻⁶. This route of infection leads to a lymphocytic choriomeningitis (LCM) which is not the result of direct cytopathic effects of the virus but is caused by the induced T-cell immune response: immunocompetent mice die whereas T-cell-deficient mice

survive³⁻⁶. By using two plaque variants of LCMV strain UBC (refs 7, 8), we found that susceptibility to LCM was dependent on the LCMV strain used ('aggressive' versus 'docile' UBC-LCMV) and on the various genes of the host mouse strains. In addition, susceptibility to LCM caused by docile UBC-LCMV was clearly linked to the murine major histocompatibility locus *H-2D*: in MHC-congenic C57BL/10 mice, susceptibility correlated with early onset and high activity of measurable LCMV-specific cytotoxic T cells in meninges and spleens and could be mapped to *H-2D*. This model shows that a severe immunopathologically mediated clinical disease in mice can be regulated directly by MHC genes of class I type and supports the notion that many MHC/disease associations directly reflect MHC-restricted and MHC-regulated T-cell reactivity⁹.

Mice of various strains and various *H-2* types were assayed for susceptibility to docile and aggressive UBC-LCMV injected i.c. at >8 weeks of age (Table 1). Susceptibility to LCMV depended on the general genetic background of the injected mice: Swiss and a few B10 mice were susceptible to both UBC-LCMV substrains; most B10 and BALB mice were susceptible to aggressive but resistant to docile LCMV; C3H and CBA mice were susceptible to docile and relatively resistant to aggressive LCMV. In B10 mice, susceptibility to LCM caused by docile UBC-LCMV was found to be regulated by *H-2*; B10.G mice with the *q* haplotype, including (B10.BR × B10.G) (*H-2^k* × *H-2^q*)F₁, were susceptible in a dominant fashion, whereas mice of haplotype *H-2^k*, *H-2^d*, *H-2^b* and *H-2^a* and most others, were resistant. *H-2*-dependence of susceptibility to LCMV was also seen in DBA mice. DBA/1 mice (*H-2^q*) were susceptible to both strains whereas *H-2* congenic DBA/2 with *H-2^d* were resistant to both aggressive and docile LCMV. In C3H mice, *H-2* did not significantly influence susceptibility independent of *H-2^q* or *H-2^k*. Swiss inbred SWR/J (*H-2^q*) mice and Swiss outbred NMRI mice (mainly *H-2^q*)¹⁰, were highly susceptible to both UBC-LCMV strains. Thus, susceptibility to LCM depends on the virus variant injected and on the multiple genes of the host that influence resistance or susceptibility (such as interferon and macrophage resistance^{11,12}), only one gene being encoded in the MHC.

The role of various *H-2* regions in susceptibility to LCM was tested in several MHC-congenic inbred strains of mice with the B10 background (Fig. 1). B10.G(*H-2^q*: *K^q*, *I^q*, *D^q*), B10.AQR(*H-2^q*: *K^q*, *I^k*, *D^d*), B10.T(6R)(*H-2^q*: *K^q*, *I^q*, *D^d*), B10.AKM(*H-2^m*: *K^k*, *I^k*, *D^q*) and B10.BR(*H-2^k*: *K^k*, *I^k*, *D^k*)

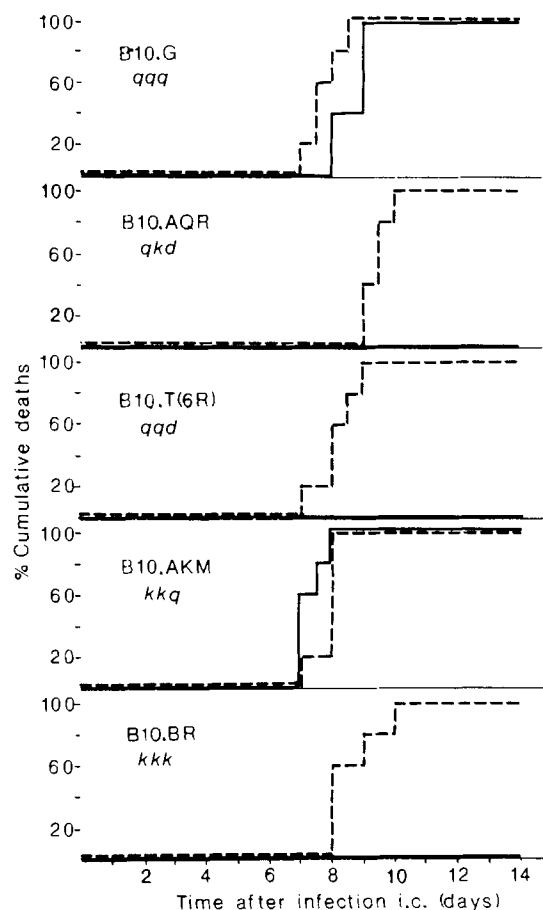


Fig. 1 Groups of five mice of various *H-2* congenic B10 murine strains were injected with ~500 PFU of docile (—) or aggressive (---) UBC-LCMV i.c. and cumulative mortality was monitored. The results shown were obtained with mice purchased from OLAC, Bicester, UK, but identical results were found with mice from the Tierspital, University of Zurich, and from Jackson Laboratory, USA. Mice were between 6 and 18 weeks old. The alleles of the *H-2K*, *I* and *D* regions are shown beneath the strain names.

Table 1 LCM mortality of various mouse strains after infection i.c. with 5×10^2 PFU docile or aggressive UBC-LCMV, assessed between day 6 and day 14

Mouse strain	<i>H-2</i>	Non- <i>H-2</i> background	% Mortality due to LCM caused by LCMV	
			Aggressive	Docile
B10.G*†	<i>q</i>	B10	100	100
B10.AKM*†	<i>m</i>	B10	100	100
B10.BR*†‡	<i>k</i>	B10	100	0
(B10.BR × B10.G)F ₁ ‡	<i>k, q</i>	B10	100	100
B10.D2‡	<i>d</i>	B10	100	0
DBA/1*†	<i>q</i>	DBA	100	100
DBA/2*†‡§	<i>d</i>	DBA	0-20	0-20
BALB/c‡	<i>d</i>	BALB	100	0
C3H.Q	<i>q</i>	C3H	20-80	60-100
C3H/HeJ‡	<i>k</i>	C3H	20-80	60-100
CBA/J‡	<i>k</i>	CBA	0-20	60-100
SWR/J*¶	<i>q</i>	Swiss	100	100
NMRI§#	<i>q</i>	Swiss	100	100

Mice (8-16 weeks old) were purchased from or given by: * OLAC, Bicester, UK; † Jackson Laboratory, Bar Harbor, USA; ‡ Tierspital, University of Zurich; § Tierversuchsinstitut Hannover, FRG; || Institut für biologisch-medizinische Forschung AG, 4414 Füllinsdorf, Switzerland; ¶ Bantin-Kingman, Aldbrough, Hull, UK; # Savo-Ivanovas, Kisslegg, FRG. The mice were infected i.c. with 30 µl of LCMV. Control mice injected with medium alone did not die.

were injected i.c. with ~500 plaque-forming units (PFU) of docile UBC-LCMV. B10.G(*H-2^q*) and B10.AKM(*H-2^m*) mice died with typical LCM whereas B10.BR, B10.AQR and B10.T(6R) mice survived. As B10.AKM(*D^q*) differs from B10.BR(*D^k*) in the *D* region of *H-2* but has in common *H-2K^k*, *I^k* and the B10 background, susceptibility to LCM is linked to *H-2D^q*. The difference in susceptibility of B10.G or B10.AKM compared with B10.BR was seen over a wide range of docile-LCMV doses between 5×10^4 PFU (the highest dose tested) and ~50 PFU i.c. (results not shown). In contrast, aggressive UBC-LCMV injected i.c. over the same dose range (results not shown, except for 500 PFU, in Fig. 1) caused death in all of the above congenic B10 mice irrespective of their MHC.

As LCMV is a T-cell-mediated immunopathological disease, the question arises of whether greater susceptibility reflects greater LCMV-specific cytotoxic T-cell activity. T-cell-mediated immune reactivity was monitored on days 5, 6 and 7 after i.c. infection with docile UBC-LCMV (Table 2, Fig. 2). We found that inflammatory cells in cerebrospinal fluid (CSF) and meninges of susceptible mice were increased from day 5 onwards, yielding values that were 5-10 times greater than those in resistant B10.BR mice on days 6 and 7. Cytotoxic activity was detectable in meningeal infiltrates and spleens of B10.G or B10.AKM mice on day 5 or 6, earlier than the barely detectable activity of B10.BR mice (day 7). The activities measured were much higher in meningeal or spleen cells from susceptible B10.G or B10.AKM than from resistant B10.BR mice (Fig. 2, Table 2).

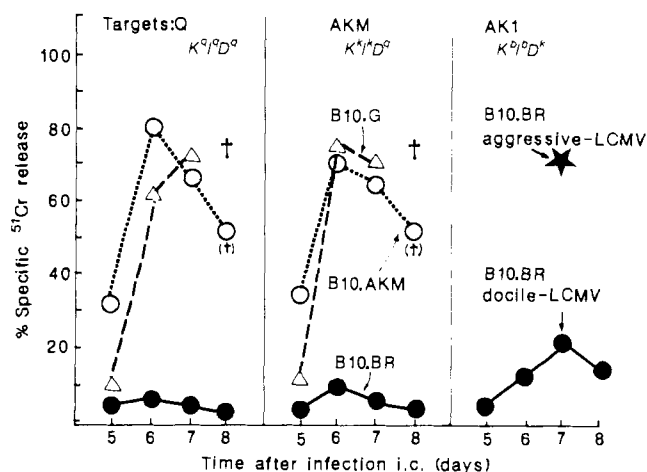


Fig. 2 Two to four B10.G(K^q, I^q, D^q) (Δ), B10.AKM(K^k, I^k, D^q) ($\circ$) and B10.BR(K^k, I^k, D^k) ($\bullet$) mice were injected i.c. with ~500 PFU of docile UBC-LCMV on days 5, 6, 7 or 8 before they were killed; all B10.G mice and one of two of the B10.AKM mice had died by day 8 ($\dagger$). The target cells used in the cytotoxicity assay were simian virus 40-transformed fibroblast lines (AKM and AK1) or a methylcholanthrene-induced tumour cell line (Q); these cells had been infected with docile UBC-LCMV for 2 days at a multiplicity of infection of ~0.01. The ^{51}Cr -release assay was run for 3.5–5 h in round-bottomed 96-well plates using 10^4 target cells; effector spleen cells were tested at various effector-to-target ratios (50, 17, 6 and 2:1) (only the results for 50:1 are shown). B10.BR spleen cells taken 7 days after immunization i.c. with aggressive UBC-LCMV were also tested on infected AK1 cells ($\star$). Immune B10.G or B10.AKM spleen cells did not induce release of ^{51}Cr from infected AK1 or L929 cells (data not shown). All effector cells were tested against YAC-1 cells, but no release greater than 7% was measured. Spontaneous release was 20–32%. $\dagger$ Indicates that all or some mice had died.

Most of the cytotoxic activity was found to be $H-2D$ -restricted. B10.G effector cells lysed infected K^q, I^q and D^q targets as efficiently as K^k, I^k and D^q targets, and B10.AKM T cells lysed D^q -compatible infected Q($H-2^q$) targets but either did not lyse or only poorly lysed K^k -compatible infected FS-9($H-2^k$) target cells. For example B10.AKM(K^k, D^q) spleens contained $2\text{--}4 \times 10^2$ lytic units (LU) when measured on infected $H-2K^k$, compared with 18 to 55×10^2 on infected D^q targets

(Table 2); this maps most of the $H-2$ -restricted cytotoxic activity to D^q , in agreement with the finding that most LCMV-specific cytotoxic T-cell activity is usually restricted to D (reviewed in ref. 6). Docile UBC-LCMV immune lymphocytes from B10.BR($H-2^k$) mice lysed infected AKM(K^k, D^q) or AK1(K^k, D^k) targets only to a small extent on days 6, 7 or 8. In contrast, those B10.BR mice that were infected with aggressive UBC-LCMV and usually died of LCMV generated high cytotoxic T-cell activity in meninges and spleen when tested on day 7 (Fig. 2, Table 2), most activity being restricted to D^k (Fig. 2)⁶. Therefore, early and high LCMV-specific MHC class I-restricted cytotoxic T-cell activity correlated positively with susceptibility to LCM and both T-cell activity and susceptibility to LCM map to $H-2D$. To our knowledge this represents the first model infection in which susceptibility can not only be shown to depend on the virus strain and on the various host genes but also to be directly regulated by classical transplantation antigens.

The clear correlation between early and high cytotoxic T-cell activity in B10.BR mice infected with aggressive as opposed to docile LCMV was not observed by Pfau *et al.* in 3–4 week-old C3H/HeJ mice tested on day 8 on infected L929 target cells¹³; this discrepancy probably represents an exception and might have been caused by the particular conditions used, that is, young age of the host and only one time point tested on L929 target cells. We found a clear correlation in 8–12-week-old C57BL/6, DBA/1, DBA/2, BALB.B and Swiss mice (R.M.Z., unpublished).

The $H-2$ -dependence of susceptibility to LCM has been studied by Oldstone *et al.*^{14,15}, who found that, on average, SWR/J($H-2^q$) mice died of LCMV to a greater extent with ~10–100-fold smaller i.c. doses of an aggressive, neurotropic LCMV (strain CA1371) than C3H/HeJ ($H-2^k$) mice. This difference in susceptibility was seen during early and shorter (days 6–10) but not longer (days 6–14) observation periods of the disease, since by day 14 there was no longer a significant difference in susceptibility¹⁴. No clear-cut linkage between susceptibility to LCM and $H-2$ has been shown since^{15,16}.

Murine LCMV represents a laboratory model in which immunocompetent mice die when infected i.c. but do not usually die if infected intravenously (i.v.) with most LCMV strains^{3–6}. The LCMV model thus demonstrates dramatically that T-cell-mediated disease is observed only if a substantial part of crucial cellular compartments, for example, leptomeningeal cells, are infected and damaged^{3–6,17,18}. Preferential infection by LCMV of leptomeninges, as opposed to other tissues or organs, enhances the lethal outcome of LCM. If frequencies of precursor

Table 2 LCMV-specific cytotoxic T-cell activity and inflammatory cells in meningeal infiltrates and spleens of mice infected i.c.

Mouse strain (<i>H-2KID</i>)	UBC-LCMV	Meningeal infiltrate							Spleen			LCMV targets used for LU determination (<i>H-2KID</i>)
		LU per brain			Lymphocyte counts ($\times 10^{-4}$)				LU ($\times 10^{-2}$) per spleen			
					per μ l CSF							
						Total cells						
		Day 5	6	7	6	7	6	7	5	6	7	
B10.BR (<i>kkk</i>)	Aggressive	NT	NT	72	NT	4	NT	80	NT	NT	32	FS-9(<i>kkk</i>)
	Docile	<5	<5	10	0.1	0.3	12	27	<1	4	2	FS-9(<i>kkk</i>)
B10.AKM (<i>kkq</i>)	Docile	<5	20	91	0.7	3.5	56	130	4	55	18	Q(<i>qqq</i>)
									<1	4	2	FS-9(<i>kkk</i>)
B10.G (<i>qqq</i>)	Docile	<5	15	160	0.4	4.2	64	160	2	40	28	Q(<i>qqq</i>)

Mice were infected with 5×10^2 PFU of aggressive or docile UBC-LCMV. On the days indicated, mice under ether anaesthesia were bled and their cerebrospinal fluid was tapped as described by Carp *et al.*²⁷; about 5 μl CSF was obtained, diluted 1:10 and counted. The skull was opened, the brain removed and both the skull and brain were rinsed with medium in a Petri dish (total cells). Single meningeal infiltrate cells and spleen-cell suspensions were prepared and assayed at ratios of 50, 17, 6 and 2:1 in a $3\frac{1}{2}$ -h test on the following ^{51}Cr -labelled docile-infected target cells: FS-9 ($H-2^k$, given by Dr H. Binz), spontaneous release 18%; and Q ($H-2^q$), 21% spontaneous release. Lytic units (LU), the number of cells giving a 33% specific ^{51}Cr -release by B10.BR effector cells on infected $H-2^k$ target cells and by B10.AKM and B10.G on $H-2^q$ target cells. LU of B10.AKM were also determined on infected FS-9 ($H-2^k$) targets. Total spleen cells varied from 5 to 10×10^7 per spleen. The results shown are for one of two similar experiments using two mice per time point. The data represent the mean values for the two mice. NT, not tested.

effector T cells are high, as is probably the case in immunologically high responders (D^q), their protective activity may limit early on the spread of LCMV to sites other than leptomeninges and therefore increase susceptibility to LCM. The differences in susceptibilities to LCM between docile and aggressive LCMV may also reflect $H-2D$ -regulated differences in cytotoxic T-cell responsiveness that correlate with possible but as yet unrecognized antigenic variations between the two substrains. In fact, Ahmed *et al.* have shown that LCMV-specific T-cell clones may distinguish between various LCMV strains that are serologically indistinguishable¹⁹.

Although the linkage of disease to the MHC has long been recognized²⁰⁻²⁵, despite much speculation, it has remained unclear how MHC-restricted T-cell recognition and MHC-encoded *Ir* gene regulation determine the MHC/disease association^{1,6,22,23}.

The observation, reported here, of a correlation between $H-2D$ -regulated T-cell activity and susceptibility to LCM suggests that many such diseases are the result of a T-cell-mediated pathophysiological mechanism⁹. In general, acute infectious agents probably do not cause disease exhibiting MHC/disease associations because such acute agents select evolutionarily for immunologically high responders in most if not all members of a species. Rather, diseases revealing MHC-linked susceptibility are usually chronic diseases of autoimmune^{24,25}, immunopathological^{6,9} or cancerous nature^{20,21}. The linkage of LCM susceptibility to a major transplantation antigen agrees with the notion⁹ that the association of disease susceptibility with the MHC is often found in connection with non- or poorly cytopathogenic infectious agents that may cause chronic infections without interfering with reproduction. These agents leave a certain amount of leeway for a balance between immune protection

and damage caused by the immune response. Accordingly, MHC-associated disease susceptibility may often signal immunological T-cell-mediated pathogenesis.

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Note added in proof: Our results are compatible with recent studies by Allan and Doherty²⁶ with BALB/c and $C-H-2^{dm2}$ mutant mice. Mutant mice expressing D^{dm2} were less susceptible to the LCMV-Armstrong isolate than BALB/c mice expressing D^d .

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Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines

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The multidrug-resistance phenotype expressed in mammalian cell lines is complex. Cells selected with a single agent can acquire cross-resistance to a remarkably wide range of compounds which have no obvious structural or functional similarities¹⁻⁶. The basis for cross-resistance seems to be a decreased net cellular accumulation of the drugs involved, and has been attributed to alterations in the plasma membrane. An over-expressed plasma membrane glycoprotein of relative molecular mass (M_r) 170,000 (P-glycoprotein) is consistently found in different multidrug-resistant human and animal cell lines, and in transplantable tumours^{1,2,6-10}. Consequently, it has been postulated that P-glycoprotein directly or indirectly mediates multidrug resistance⁷⁻¹¹. Here we report the cloning of a complementary DNA encoding P-glycoprotein. Southern blot analysis of hamster, mouse and human DNA using this cDNA as a probe showed that P-glycoprotein is conserved and is probably encoded by a gene family, and that members of this putative family are amplified in multidrug-resistant cells.

A cDNA library was constructed using the expression vector λ gt11 (ref. 12) and messenger RNA isolated from the highly multidrug-resistant cell line, CH^RB30 (see accompanying article)⁶. Plaques were selected by probing with a P-glycoprotein-specific monoclonal antibody, C219 (ref. 6). One

of the presumptive P-glycoprotein cDNA clones, containing an insert of ~600 base pairs (bp), was selected for further study. This cDNA, called λ CHP1 when in the λ gt11 vector, or pCHP1 when subcloned into the pUC9 vector¹³, was transcribed into a 140,000- M_r *lacZ* fusion protein in λ CHP1-infected bacterial cells (Fig. 1A). The size of this fusion protein relative to the uninterrupted β -galactosidase suggested that most of the λ CHP1 insert contained P-glycoprotein coding sequence. Strong evidence that the cDNA insert coded for P-glycoprotein sequences is provided in Fig. 1B, where the same *lacZ* fusion protein was recognized by monoclonal antibodies that identify three independent epitopes of P-glycoprotein⁶.

Further corroboration that the cloned cDNA fragment encodes P-glycoprotein was provided by Northern blot analysis of the highly drug-resistant cell line CH^RB30 and of its sensitive parental cell line, AuxB1 (Fig. 2A). The pCHP1 insert hybridized to a single mRNA component of ~4.7 kilobases (kb), which was strongly expressed in CH^RB30. Slot-blots of mRNA isolated from a series of increasingly multidrug-resistant Chinese hamster ovary (CHO) cells, and from a drug-sensitive revertant cell line (Fig. 2B) revealed hybridization signals consistent with the levels expected if pCHP1 sequences encode a portion of P-glycoprotein. Both the correlation of mRNA expression with multidrug resistance and the size of this mRNA species were consistent with that expected for P-glycoprotein².

Southern blot analysis of multidrug-resistant hamster, mouse and human cell lines and of their drug-sensitive parental cell lines (Fig. 3) demonstrated four main points: (1) in all cases, amplification of pCHP1-homologous sequences correlated with expression of multidrug resistance. The degree of amplification increased with increasing drug resistance in the CHO series, and was lost in the CHO revertant, I10. Similar amplification of pCHP1-homologous sequences has also been detected in a daunorubicin-resistant CHO cell line⁷ (data not shown), and in the adriamycin-resistant Chinese hamster lung cell line LZ¹⁴ (J. H. Gerlach and V.L., unpublished observations). Roninson *et al.*¹⁴ have previously observed 55 kilobase pairs (kbp) of amplified DNA fragments common to both LZ and CH^RC5

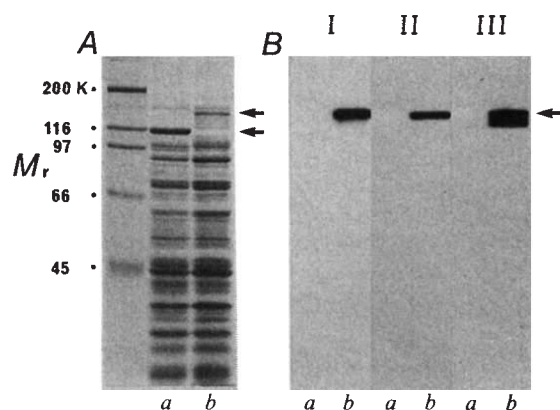


Fig. 1 β -Galactosidase fusion protein containing a P-glycoprotein-specific cDNA insert. **A**, SDS-polyacrylamide gel electrophoresis (PAGE) of aliquots containing 75 μ g protein from lysates of Y1089 bacteria infected with λ gt11 (**a**) or λ CHP1 (**b**). M_r markers in the extreme left-hand lane were as described previously⁹. Arrows indicate the *lacZ* fusion product of λ CHP1 (top arrow) and uninterrupted β -galactosidase (bottom arrow). **B**, Western blots of lanes shown in **A**. Monoclonal antibodies⁶ recognizing three different epitopes on the P-glycoprotein molecule were used. Three identical blots were probed with representative monoclonal antibodies of group I (C219 probe), group II (C32 probe) and group III (C494 probe), as indicated.

Methods. The cDNA probe, λ CHP1, was obtained as follows. Poly(A)⁺ RNA was isolated from the highly colchicine-resistant cell line, CH^RB30, by standard techniques^{22,23}. cDNA was synthesized and cloned into the β -galactosidase-encoding *lacZ* gene of the bacteriophage λ gt11 (ref. 12) by the methods of Huynh *et al.*²⁴. The library contained 1.3×10^6 recombinants, 65% of which contained cDNA inserts. Screening was done with radioiodinated ($\sim 10^6$ Ci μ g⁻¹) monoclonal antibody C219. After plaque purification, the insert (~ 600 base pairs, bp) from one of the positive clones, λ CHP1, was subcloned in the plasmid pUC9 (ref. 13). This recombinant, designated pCHP1, was used in subsequent hybridization analyses. Infection of Y1089 bacteria with either λ gt11 or λ CHP1 and induction of the *lacZ* products with isopropyl β -D-thiogalactopyranoside were done as described¹². Bacteria were lysed by brief sonication, solubilized and then subjected to SDS-PAGE according to Laemmli²⁵. Western blot analyses were done according to Towbin *et al.*²⁶, as described previously⁹.

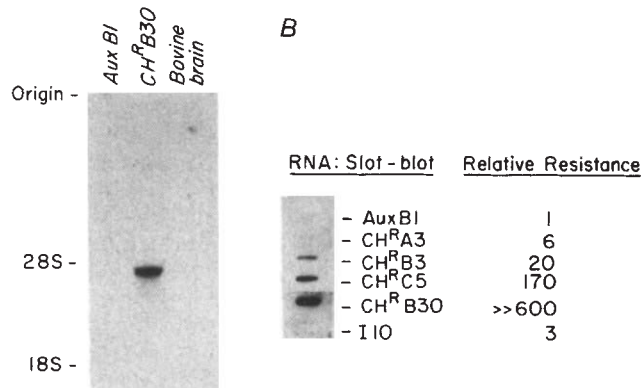


Fig. 2 RNA analysis using the pCHP1 probe. **A**, Northern blots of total cellular RNA from drug-sensitive AuxB1 and highly multidrug-resistant CH^RB30 cells. The third lane contained RNA from normal bovine brain as a control. **B**, Slot-blot analysis of RNA from a series of CHO cells with different levels of drug resistance. The relative resistance to colchicine is indicated opposite each slot^{1,2}. The clonal lines CH^RA3, CH^RB3 and CH^RC5 were derived from sequential selections for increasing colchicine resistance; CH^RB30 was obtained by continuous culture of CH^RC5 in increasing colchicine concentrations from 5 to 30 μ g ml⁻¹, and revertant I10 was selected from CH^RC5 in a single step^{2,6}. The level of P-glycoprotein expression has been shown to correlate with the degree of drug resistance^{6,9}. Northern and slot blots were performed as described by Thomas²⁷. The probe used was pCHP1 insert, nick-translated to a specific activity of $\sim 10^8$ d.p.m. μ g⁻¹.

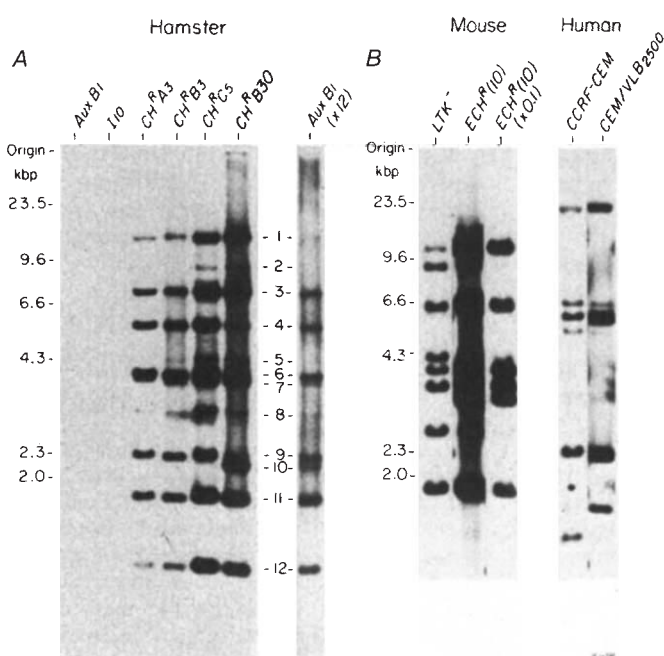


Fig. 3 DNA analysis with the pCHP1 probe. Southern blots of *Eco*RI-digested genomic DNA from: **A**, the series of increasingly multidrug-resistant CHO cells shown in Fig. 2; and **B**, multidrug-resistant mouse and human cells. Multiple bands were also observed when other restriction enzymes were used with CHO DNA (not shown). Note the appearance of new bands, probably 'novel joints'¹⁵, not detected in sensitive parental cells (for example, band 2 in CH^RC5 and CH^RB30). Quantitative densitometry of the CH^RB30 band pattern in a separate experiment revealed that restriction fragments 4, 6 and 9 were amplified 10–20-fold, while fragments 7, 10, 11 and 12 were amplified by 50-fold or more.

Methods. DNA was digested with *Eco*RI and electrophoresed in 0.6% agarose gels. Transfer to nitrocellulose was performed essentially as described by Southern²⁸. Hybridization was in 50% formamide, 5 $\times$ SSC, 20 mM sodium phosphate pH 6.5, 200 μ g ml⁻¹ sonicated salmon sperm DNA, 0.1% bovine serum albumin (BSA), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 10% dextran sulphate, plus 10^6 d.p.m. ml⁻¹ pCHP1 insert nick-translated to a specific activity of $\sim 10^8$ d.p.m. μ g⁻¹. Filters were washed twice for 5 min each in 2 $\times$ SSC, 0.1% SDS at room temperature, twice for 30 min in the same solution plus 0.1% sodium pyrophosphate at 50 $^{\circ}$ C, and twice for 30 min in 0.1 $\times$ SSC, 0.1% SDS and 0.1% sodium pyrophosphate at 50 $^{\circ}$ C. M_s were determined from *Hind*III-digested λ DNA, which was co-electrophoresed. Hamster cell lines were those described in Fig. 2 legend. The resistant mouse cell line was derived from L cells and was selected with colchicine¹⁰. CEM/VLB₂₅₀₀ is a highly resistant human leukaemic cell line selected for growth in 2,500 ng ml⁻¹ vinblastine²⁰, from the resistant subline CEM/VLB₁₀₀ (ref. 4). CCRF-CEM is the drug-sensitive parental cell line. Autoradiographs of hamster DNA were for 15 h, except for the lane marked AuxB1 ($\times 12$), which was exposed 12 times longer. Autoradiographs of mouse and human DNA were for 7 days, except the lane marked ECH^R(10) ($\times 0.1$), which was exposed for one-tenth of that time. Band 1 in AuxB1 is under-represented in this experiment.

using a DNA renaturation technique in agarose gels; in view of our present findings, the P-glycoprotein genes probably comprise a major portion of those sequences.

(2) The pCHP1 insert detected multiple bands in the Southern blots of *Eco*RI-digested DNA, despite the fact that this cDNA fragment was only ~ 600 bp long and contained no internal *Eco*RI sites. Although other explanations are possible, the simplest is that P-glycoprotein is encoded by a multigene family. In support of this notion, only a subset of the 10 bands detected in CHO cells were transfected (and these were amplified) in mouse cells transformed to the multidrug-resistant phenotype with DNA derived from multidrug-resistant hamster cells R.-p. Du, K.D. and V.L., unpublished observations).

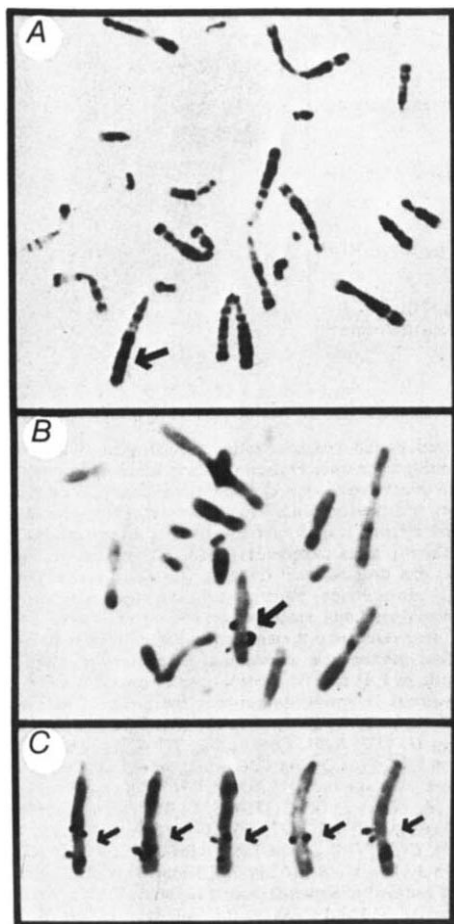


Fig. 4 Cytological evidence for localization of amplified P-glycoprotein sequences to a homogeneously staining region (HSR) on the Z4 chromosome of CH^RB30. **A**, Representative G-banded CH^RB30 cell demonstrating an HSR on chromosome Z4 (arrow). The most consistent and characteristic chromosomal alteration between the parental line AuxB1 and the highly colchicine-resistant CH^RB30 line was the presence of an HSR on the long arm of chromosome Z4. **B**, Localization of autoradiographic grains to the Z4 HSR (arrow) following hybridization *in situ* with ³H-labelled pCHP1. **C**, Examples of grain localization to the Z4 HSR (arrows) from five different CH^RB30 cells as in **B**. Of 25 cells examined, 20 yielded one or more autoradiographic grains localized to the Z4 HSR, and 45% (60/133) of all grains were localized to the Z4 HSR. No significant grain localization in the drug-sensitive cell AuxB1 was observed in the same conditions (data not shown). **Methods.** G-banding and *in situ* hybridization analysis were performed as described previously^{29,30}. Cells were hybridized for 15.5 h at a final DNA concentration of 0.65 µg ml⁻¹, followed by exposure for 7 days before autoradiographic development (**B**, **C**). The autoradiographic analysis was performed with unbanded chromosomes; however, the size and arm length ratio of the Z4 HSR allowed it to be unequivocally identified in CH^RB30 cells.

(3) The Southern blots also indicate that the presumptive CHO P-glycoprotein gene family appears to be clustered within one amplification unit (an amplicon), because in CH^RA3, which was selected in a single step from the sensitive parent AuxB1, all the restriction fragments were simultaneously amplified by ~10–20-fold. Coordinate amplification did not occur in mouse and human cell lines, suggesting that the P-glycoprotein genes detected in these cases were not part of the same amplicon. The appearance of amplified fragments that have no counterparts in parental DNA may represent novel joints. Such regions are found in tandemly amplified sequences¹⁵.

(4) The ability of the pCHP1 insert to detect human sequences under stringent conditions provided evidence that P-glycoprotein is conserved at the DNA level, as well as at the protein level⁶.

Karyotypic markers of gene amplification—homogeneously staining regions or double minute chromosomes—have been described in multidrug-resistant cell lines^{3,11,16–19}. In the CHO cell system, there exists an extensive homogeneously staining region on the Z4 chromosome in CH^RB30 cells (Fig. 4) that is not found in the drug-sensitive parental cell line. *In situ* hybridization using ³H-labelled pCHP1 revealed that the amplified P-glycoprotein sequences reside within the Z4 homogeneously staining region. No significant grain localization to other chromosomes was apparent.

The fact that multidrug resistance is associated with gene amplification suggests that the basis of this phenotype is an increased expression of a normal gene product. It may be speculated that the function of P-glycoprotein in normal cells is to modulate cellular permeability to diverse compounds and that overexpression of P-glycoprotein in multidrug-resistant cells results in an exaggeration of this normal function. The present study raises the possibility that different members of the putative P-glycoprotein gene family control different aspects of permeability, and that the sum of their individual effects produces the diversity of the multidrug-resistance phenotype. Differential expression of members of this family may also result in the variability in resistance patterns among different multidrug-resistant cell lines. The cloning and transfection of a functional P-glycoprotein gene will provide insight into these hypotheses and will determine whether or not overexpression of P-glycoprotein alone is sufficient to mediate the multidrug-resistance phenotype.

Recently, we have detected P-glycoprotein overexpression in some cases of advanced ovarian cancer²⁰. This suggests that the multidrug-resistance mutation can occur in human malignancies and result in non-responsiveness of the malignancy to combination chemotherapy^{6,20,21}. Further studies are required to determine the generality of this finding among other malignancies. The homology of pCHP1 to human P-glycoprotein sequences should facilitate studies in this area.

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Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies

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One reason for the failure of chemotherapy in the treatment of advanced cancers may be the outgrowth of multidrug-resistant tumour cells¹⁻⁵. Multidrug resistance has been modelled in numerous mammalian cell lines in which the phenotype is characterized by a pleiotropic cross-resistance to unrelated drugs¹⁻⁷. In the study reported here, we have produced monoclonal antibodies whose binding to plasma membranes of different multidrug-resistant mammalian cells correlates with the degree of drug resistance. All these antibodies are specific for P-glycoprotein, a cell surface component of relative molecular mass (M_r) 170,000 (170K) that has been described previously⁸⁻¹⁵, and are directed against three spatially distinct epitopes which define a conserved cytoplasmic domain in the C-terminal region of the P-glycoprotein polypeptide. The conserved nature of P-glycoprotein and its low-level expression in drug-sensitive cells suggest that it has an important function at the cell surface. The monoclonal antibodies against P-glycoprotein described here might serve as diagnostic reagents for clinically unresponsive tumours¹⁶.

Hybridomas were produced from splenocytes of mice immunized with SDS-solubilized plasma membranes of multidrug-resistant Chinese hamster ovary (CHO) and human cell lines. Primary screening of the hybridomas was achieved using nitrocellulose test strips spotted with SDS-solubilized plasma membranes of drug-sensitive and multidrug-resistant CHO and human cells (for details see Fig. 1 legend). By this means we were able to select hybridomas secreting monoclonal antibodies specific for drug resistance-associated antigens in either CHO or human cells. Screening with denatured antigens immobilized on nitrocellulose ensured that the monoclonal antibodies selected would be of use in the detection of antigens in protein immunoblots (Western blots). Eight stable hybridoma clones were ultimately isolated, yielding three monoclonal antibodies which appeared to be specific for resistance-associated CHO antigens, and five that bound antigens common to multidrug-resistant CHO and human plasma membranes.

To characterize the above differences in binding specificity more precisely, supernatants from cloned hybridomas were screened with a larger panel of plasma membranes from drug-sensitive and multidrug-resistant cell lines (Table 1). The results (Fig. 1) showed that the monoclonal antibodies could be classified into three distinct groups according to the pattern of staining obtained in the dot-blot panel. No significant staining of drug-sensitive cell membranes was observed with any of the eight monoclonal antibodies. The antibodies in group I stained membranes of all drug-resistant cells tested, while those of group II failed to stain drug-resistant human membranes. The single member of group III stained drug-resistant CHO and human membranes, but not those of mouse. Further evidence of distinct epitopes was provided by competitive binding assays, as shown in Fig. 2. The latter results clearly allowed the classification of the monoclonal antibodies into three groups (I, II and III) on the basis of their binding to three spatially distinct epitopes.

The antigen specificity of each of the monoclonal antibodies was determined by probing plasma membrane components in Western blots. Figure 3A shows the results for plasma membranes from various cell lines (Table 1). Bands of $M_r \sim 170K$ were stained in lanes representing CHO cells of increasing colchicine resistance (Fig. 3A, lanes b-d), and a daunorubicin-resistant CHO cell line (lane e). Similar results were obtained

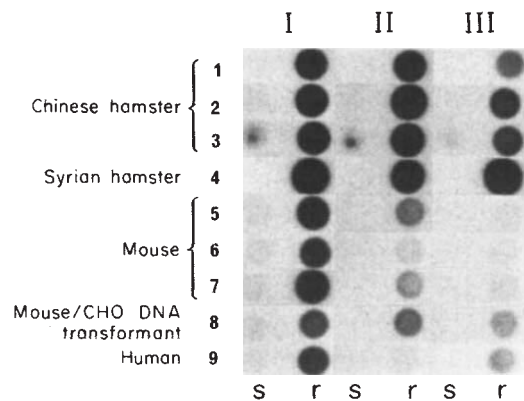


Fig. 1 Selection and characterization of monoclonal antibodies, using immunoblotting techniques. Dot immunoblots were used in a primary screening assay to select hybridomas of interest (see below). Shown here are dot immunoblots which were used in a more comprehensive secondary screening of re-cloned hybrids. Eight hybridomas were tested against a panel of SDS-solubilized plasma membranes purified from pairs of matched drug-sensitive (s) and drug-resistant (r) cells; 2 μ l (2 μ g protein) of each were spotted onto nitrocellulose filters. Hybridoma supernatants were adjusted to a monoclonal antibody concentration of 1 μ g ml⁻¹. Note the differential staining of drug-resistant cell membranes. Three different staining patterns were observed and the eight monoclonal antibodies were classified accordingly into groups I, II and III. Comparison of rows 4, 5 and 9 emphasizes major differences in specificity between the groups. The assignment of monoclonal antibodies to groups was as follows. Group I: C11, C26, C36, C219; group II: C32, C103, C699; group III, C494. Their isotypes were $\kappa\gamma 2a$, except for C11, C26 and C36, which were $\kappa\gamma 1$. Test cell lines were (sensitive line, resistant line): (1) AuxB1, CH^RB30; (2) AuxE29, DNR^R159; (3) CHEF 18, VCR 1.5; (4) C1₂TSV₅S, C1₂TSV₅R₂; (5) LMTK⁻, ECH^R; (6) S180, S180A5; (7) UV-2237, UV-2237-ADM^R; (8) LTA, LC5B1; (9) CCRF-CEM, CEM/VLB₅₀₀ (see Table 1 for description of cell lines).

Methods. Twelve-week-old (BALB/c $\times$ C3H)F₁ mice were immunized with injections of purified plasma membranes isolated according to Riordan and Ling¹⁰ from CH^RB30 and CEM/VLB₅₀₀ cell lines. Membrane protein was assayed according to a modification³² of the method of Lowry *et al.*³³. Membranes were solubilized in a 2% SDS-containing buffer as for SDS-PAGE^{11,12}, with a protein/SDS ratio of 1:1.4, and were emulsified 1:1 with Freund's incomplete adjuvant. The first injection consisted of 200 μ g of CH^RB30 membrane given intraperitoneally, followed after 14 days by a second, identical injection. After 14 months the mice were injected with a mixture of 100 μ g each of CH^RB30 and CEM/VLB₅₀₀ plasma membranes. Identical injections were given 14 and 21 days after the first injection in this series. In addition, on day 21 a mixture containing 100 μ g of sonicated plasma membrane vesicles from each of CH^RB30 and CEM/VLB₅₀₀ was injected intravenously. On day 26, spleens were removed aseptically and splenocytes fused 3:1 with the Sp2/0-Ag14 fusion partner^{34,35}. Cells were plated in 96-well plates at 10⁵ myeloma cells per well in RPMI-1640 medium containing 100 μ M hypoxanthine, 0.5 μ M aminopterin, 30 μ M thymidine, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol and 15% fetal bovine serum. When wells seemed to be 25% confluent (50–80% of wells after 10–14 days), they were screened for specific antibody-producing hybrids. Primary screening of individual hybridoma supernatants was done using a panel of SDS-solubilized plasma membranes, by dot blotting. Test strips were prepared by spotting 1 μ l of SDS-solubilized plasma membrane (1 mg ml⁻¹ protein; prepared as for SDS-PAGE^{11,12}) onto numbered 5 $\times$ 25 mm nitrocellulose strips. Each strip was spotted with plasma membranes from the drug-sensitive CHO cell line AuxB1, the drug-resistant CHO line CH^RB30, the drug-sensitive human cell line CCRF-CEM, and the drug-resistant human line CEM/VLB₅₀₀ (see Table 1 for description of cell lines). The dried dot blots were blocked in 3% bovine serum albumin (BSA)/saline³⁶ for 1 h at 37 $^{\circ}$ C, then placed in test tubes containing hybridoma supernatants diluted 1:10 in BSA/saline. After overnight incubation at 4 $^{\circ}$ C, the strips were washed in several changes of phosphate-buffered saline (PBS), then developed with ¹²⁵I-labelled IgG goat anti-mouse immunoglobulins (Cappel; 10⁶ c.p.m. ml⁻¹ in 3% BSA/saline) for 6 h at 25 $^{\circ}$ C. After washing and drying, the test strips were exposed overnight, with intensifying screens, on Kodak X-AR5 film at -70 $^{\circ}$ C. About 1,000 strips were processed simultaneously. Candidate wells were expanded and cloned twice by limiting dilution. The above screening method was repeated to pick positive clones. Secondary screening, as shown, was done in a similar manner, but using plasma membranes derived from several different cell lines. Eight stable clones were finally isolated.

when blots of colchicine-resistant mouse cells (Fig. 3A, lane h) and vinblastine-resistant human leukaemic cells (lane j) were probed (compare with drug-sensitive parental cells in lanes a, g and i, and a drug-sensitive revertant, lane f). All three classes of monoclonal antibody stained bands of identical M_r when a

side-by-side comparison of Western blots was made (data not shown). Previous work has established that homologous P-glycoproteins with a consistent M_r of 170K are expressed in all the multidrug-resistant cell lines tested here¹¹⁻¹⁵. Longer exposure of the blots (Fig. 3A, lanes *k-n*) revealed faint bands

in the membranes of drug-sensitive parents and revertant cells in lanes *a, f, g* and *i*, respectively. Low-level expression of P-glycoprotein in drug-sensitive cells has been reported previously¹². Trivial explanations for the apparent homologies of the 170K bands in different species, such as detection of pros-

Table 1 Description of cell lines

	Parental line	Resistant line	Drug of selection	Selection conc. ($\mu\text{g ml}^{-1}$)	Relative resistance	Cross-resistance	Ref.
Chinese hamster	AuxB1	CH ^R A3	CCH	0.10	7	PUR ETB DNR CCH ERY	23, 24
		CH ^R B3	CCH	3.0	31	PUR ETB DNR CCH EME ACR	23, 24
		CH ^R C5	CCH	10	145	CCH GRD PUR DNR EME VLB DOX	23, 24
		CH ^R B30	CCH	30	640	CMD ACR ETB CYB ERY PRO	*
		DNR ^R 51	DNR	0.50	41	CCH PUR DOX	11
		DNR ^R 159	DNR	1.0	37	DNR PUR DOX CCH VLB EME	11
	AuxE29	CP-3.7	PHA	—	—	VLB DNR PUR DOX CCH	14
		I10	—	—	2	CCH PUR DNR	25
		VCR 1.5	VCR	1.5	40	CCH CMD VLB DNR PUR	26
	CHEF 18	CL ₂ TSV ₅ R ₂	DAC	2.0	1,750	VCR PUR CMD	27, 28
	LMTK ₂	ECH ^R	CCH	0.50	26	DAC PUR PRO	15
Syrian hamster	UV-2237	UV-2237-ADM ^R	DOX	1.0	140	CCH PUR CYB DAC EME	13, 29
	S180	S180A5	DOX	~1	40	VCR DNR VLB MMC AMS DAC ADX	30
	LTA	LC5B1	CCH	0.50	43	DNR MRM ADX VCR DAC	15
Mouse/CHO DNA transformant	CCRF-CEM	CEM/VLB ₁₀₀	VLB	0.10	70	PUR CCH EME CYB DAC	31
		CEM/VLB ₅₀₀	VLB	0.50	~400	(VCR VLB PUR CCH DNR EME)*	*
		CEM/VLB ₁₀₀₀	VLB	1.0	~800	NA	*
		CEM/VLB ₅₀₀₀	VLB	5.0	~4,000	VCR VLB PUR DNR DOX	*

Cell lines of the series CH^RA3, CH^RB3 and CH^RC5 were clonally selected, stepwise, from AuxB1 (ref. 23). CH^RB30 was selected by continuous culture, in increasing colchicine (CCH) concentrations, from CH^RC5. CP-3.7 is a phytohaemagglutinin (PHA)-resistant mutant, selected from CH^RC5, which retains the multidrug resistance phenotype of CH^RC5 (ref. 14). I10 is a revertant cell line, selected in a single step from CH^RC5 (ref. 25). CEM/VLB₁₀₀ was obtained from Dr W. T. Beck³¹ and was cloned in methylcellulose containing 0.1 $\mu\text{g ml}^{-1}$ vinblastine (VLB). The series of VLB-resistant human leukaemic cell lines, CEM/VLB₅₀₀, CEM/VLB₁₀₀₀ and CEM/VLB₅₀₀₀, was derived from a clone of CEM/VLB₁₀₀ by continuous growth in increasing drug concentrations and selection at 0.5, 1.0 and 5.0 $\mu\text{g ml}^{-1}$ vinblastine, respectively. Relative resistance was determined as described previously²⁴. Relative resistance values for the CH^R CHO series and CEM/VLB series cells were re-assayed for the present work and may not agree exactly with previously reported values. Drugs that were tested for cross-resistance are listed in order of decreasing relative cross-resistance. ACR, acriflavine; ADX, AD-32; AMS, amsacrine; CMD, colcemid; CYB, cytochalasin B; DAC, actinomycin D; DNR, daunorubicin; DOX, adriamycin; EME, emetine; ERY, erythromycin; ETB, etidium bromide; GRD, gramicidin D; MMC, mitomycin C; MRM, marcellomycin; PRO, proflavine; PUR, puromycin; VCR, vincristine. NA, not assayed.

* Present study.

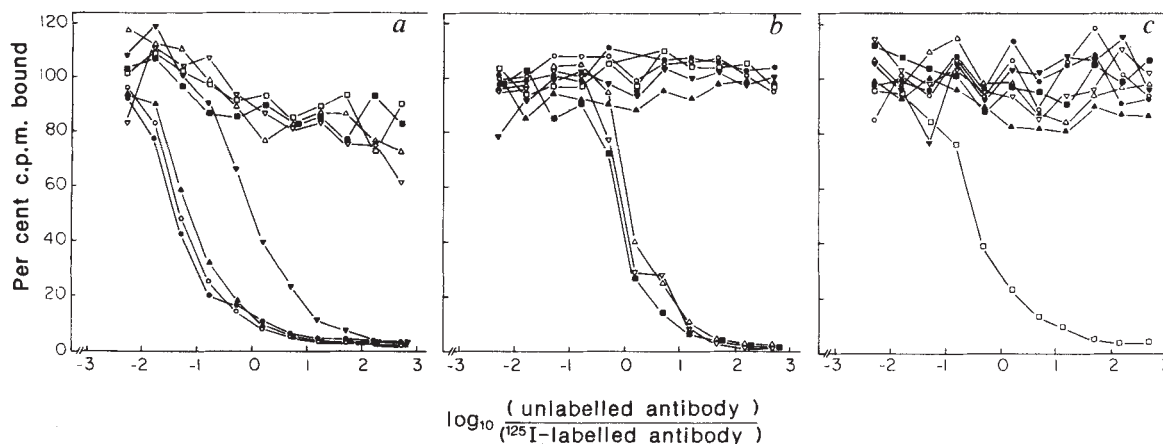
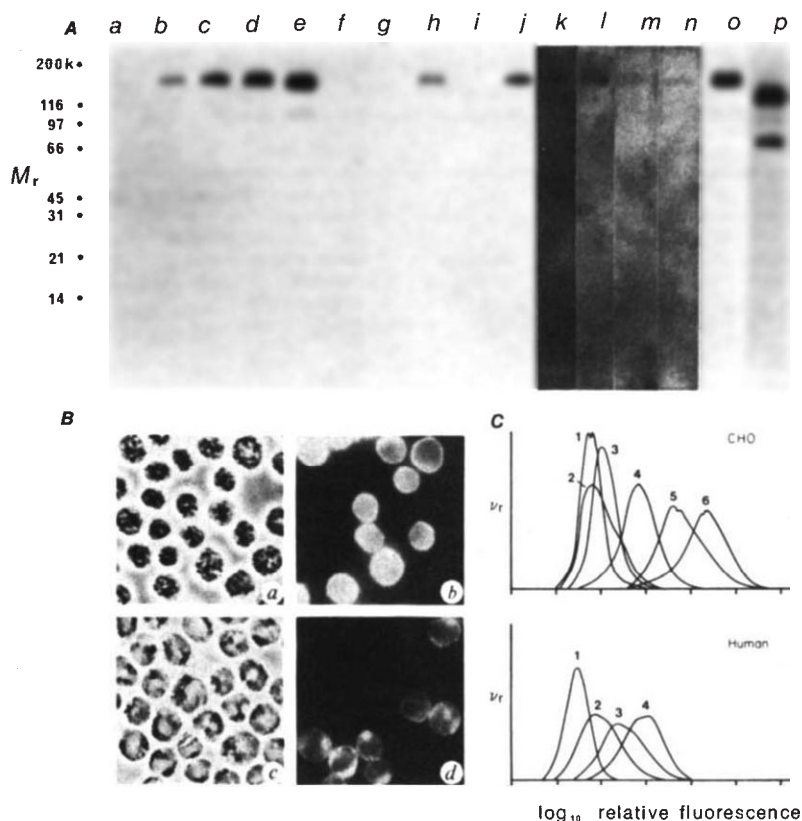


Fig. 2 Direct competitive binding assay of eight monoclonal antibodies against purified, radioiodinated antibody. Target antigen was plasma membrane vesicles from CH^RB30, immobilized on nitrocellulose in 96-well plates. The abscissa is a logarithmic scale of the ratio of the concentrations of unlabelled competing antibody and radiolabelled antibody. The ordinate shows the percentage of c.p.m. bound, normalized to c.p.m. bound in the absence of competing antibody. Results of single simultaneous experiments are shown for clarity. A, Competition between a labelled group I monoclonal antibody (C219) and dilutions of eight different ascites fluids. No group II or III antibodies competed with C219. The shift in the competition curves of other group I antibodies with respect to the self-competition curve of C219 probably reflects differences in binding affinity. B, Competition between the group II antibody C32 and other ascites fluids. Monoclonal antibodies of groups I and III did not compete with C32. The binding kinetics of the three group II antibodies appeared to be similar. C, Competition data for the single representative of group III (C494). No group I or group II antibodies competed with C494. The monoclonal antibodies tested were: ○, C11; ●, C26; △, C32; ▲, C36; ▽, C103; ▼, C219; □, C494; and ■, C699.

Methods. Millititre HA plates (Millipore) were coated with 1 μg of CH^RB30 plasma membrane in 30 μl PBS per well, and incubated overnight at 37 °C. Wells were blocked with 100 μl of 10% BSA in PBS for 4 h at 37 °C. After washing with PBS, the wells were incubated with 30 μl of radioiodinated, purified monoclonal antibody (20 ng, 100,000 c.p.m.) mixed with 30 μl of ascites fluid serially diluted threefold in 10% BSA/PBS. After incubation for 16 h at 25 °C, the plates were washed with PBS containing 0.1% Tween 20. Dried wells were punched out, and bound ¹²⁵I-labelled antibody was quantitated by γ -counting. Monoclonal antibodies were purified by chromatography on protein A-Sepharose (Pharmacia) and concentrated by ammonium sulphate precipitation³⁷. Purified antibodies were radioiodinated using chloramine T³⁸. C219 protein was quantitated gravimetrically ($\pm 5\%$) by lyophilizing the purified antibody and drying to constant weight, *in vacuo* at 25 °C. Other purified monoclonal antibodies were quantitated by absorbance at 280 nm in PBS against C219 standards. Clarified ascites were titred by spotting twofold serial dilutions onto nitrocellulose and probing with radiolabelled goat anti-mouse immunoglobulins. Spots were compared by autoradiography and densitometry, using purified C219 as a standard. Ascites of Sp2/0-Ag14 cells gave no significant background.

Fig. 3 Specific staining of P-glycoprotein in Western blots and in cells. **A**, Western blots of plasma membranes of drug-sensitive and resistant cells (Table 1), using monoclonal antibody. A group I antibody (C219) was used throughout this figure. Lanes *a*–*d*, CHO cells of increasing drug resistance: *a*, AuxB1; *b*, CH^RB30; *c*, CH^RB3; *d*, CH^RC5; *e*, daunorubicin-resistant CHO cell line, DNR^R51; *f*, drug-sensitive revertant of CH^RC5, I10; note that the staining intensity of the 170K P-glycoprotein band correlates with the degree of drug resistance (Table 1); *g*, *h*, sensitive and colchicine-resistant mouse cells (LMTK⁺, ECH^R); *i*, *j*, sensitive and vinblastine-resistant human cells (CCRF-CEM, CEM/VLB₁₀₀); *k*–*n*, eightfold longer exposures of lanes *a*, *f*, *g* and *i*, respectively, showing staining of the 170K band in membranes of drug-sensitive cells; lane *o*, same loading as in *d*, but the blot was treated with bacterial alkaline phosphatase (10 U ml⁻¹ in 10 mM Tris-HCl (pH 8.0), 2 h at 25 °C) according to Davis *et al.*³⁹ before probing with monoclonal antibody. Phosphatase treatment did not affect antibody binding (compare with lane *d*). The same result was obtained when the plasma membrane sample was treated with phosphatase before SDS-PAGE. *p*, CP-3.7, showing that depletion of *N*-linked carbohydrate¹⁴ on P-glycoprotein (note the shift in *M_r*) did not affect antibody binding. Minor bands of lower *M_r* are probably degradative fragments of the major band. SDS-PAGE according to Fairbanks *et al.*⁴⁰ and Western blots according to Towbin *et al.*²⁶ were performed as described previously^{11,12}, using 50 µg of membrane protein per lane. Protein staining of gels (not shown) gives a complex pattern of at least 30 bands^{11,12}. **B**, Indirect immunofluorescence staining of drug-resistant cells with monoclonal antibody. Mixtures of cell were stained with C219 and fluorescent second antibody: *a*, phase-contrast photomicrograph of a 1:1 mixture of AuxB1 and CH^RB30 cells; *b*, fluorescence photomicrograph of same field as in *a*; *c*, phase-contrast photomicrograph of a 1:1 mixture of CCRF-CEM and CEM/VLB₅₀₀₀ cells; *d*, fluorescence photomicrograph of same field as in *c*. AuxB1 or CCRF-CEM cells alone (not shown) showed no significant fluorescent staining in these conditions. Fields of CH^RB30 or CEM/VLB₅₀₀₀ cells alone (not shown) showed similar staining of all cells. **C**, Flow cytometry of indirect immunofluorescence staining using cell lines of different drug resistance. The abscissa is a logarithmic scale of fluorescence intensity relative to calibration beads (see below). The ordinate shows the relative frequency (*ν_r*) of cells in a given fluorescence intensity channel. Typically about 10,000 cells were analysed in 256 channels for each curve. The top panel shows a series of CHO cell lines, the bottom panel a series of human cell lines. Peaks are identified as follows. Top panel: 1, AuxB1; 2, I10; 3, CH^RA3; 4, CH^RB3; 5, CH^RC5; 6, CH^RB30. Bottom panel: 1, CCRF-CEM; 2, CEM/VLB₁₀₀; 3, CEM/VLB₅₀₀; 4, CEM/VLB₅₀₀₀. Staining was done with the monoclonal antibody C219, and fluorescein-conjugated second antibody (see below). Note the correlation between the degree of drug resistance (Table 1) and fluorescence staining intensity. **Methods.** For fluorescence microscopy, cells were fixed in 0.46% formaldehyde in PBS (pH 7.0), on ice for 2–3 min, after which they were washed twice with PBS at 4 °C and fixed further in 95% ethanol at –20 °C for 5 min, then in acetone at –20 °C for 5 min. Cells were pelleted at 4 °C, the acetone was aspirated and the pellet air-dried for 5 min before staining. For flow cytometry, cells were fixed only in 70% methanol at –20 °C for 5 min, and washed twice in PBS before staining. Cells (10⁶ ml⁻¹) were stained in 1% normal rabbit serum (NRS)/PBS containing monoclonal antibody (~3 µg ml⁻¹) from diluted ascites for 1 h at 4 °C with inversion. Cells were washed twice with 1% BSA/PBS, then incubated in fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dako) diluted 50-fold in 1% NRS/PBS for 1 h at 4 °C. Cells were washed three times in 1% BSA/PBS and resuspended in 100 µl of 50% glycerol in PBS containing 20 mg ml⁻¹ 1,4-diazabicyclo(2,2,2)octane. Stained cells were viewed and photographed using a Zeiss epifluorescence microscope fitted with an H485 filter pack. For flow cytometry, cells were resuspended to 10⁶ ml⁻¹ in 1% BSA/PBS and analysed in a Coulter EPICS V flow cytometer. The logarithm of integrated fluorescein isothiocyanate fluorescence was determined with forward-angle light scatter gated to analyse only single cells. Approximately 10⁴ cells were analysed per cell line. Fluorescence intensity was standardized against Coulter 10 µm Full Bright calibration beads at 10-fold and 100-fold attenuation.



thetic groups like phosphate, or common carbohydrate epitopes, are excluded by the controls in Fig. 3A, lanes *o* and *p*: here the absence of phosphate or *N*-linked carbohydrate moieties, respectively, had no apparent effect on binding of antibody to the antigen. Based on the correlation of increased expression of the antigen with increased multidrug resistance, and the conservation of structure of the antigen irrespective of the drug of selection in a number of mammalian species, we concluded that the antigen bound by each of the monoclonal antibodies was P-glycoprotein, as defined previously¹².

In many diagnostic and research applications, it is important for an antibody to be capable of detecting the antigenic site as it is presented in cells and tissues. In this regard we have found, using both radiolabelled monoclonal antibodies and indirect immunofluorescence staining, that none of the monoclonal antibodies binds the surface of intact multidrug-resistant cells (data not shown). Nevertheless, as shown in Fig. 3B, using fixed and permeabilized cells, the monoclonal antibodies labelled the surface membrane of resistant CHO and human cells, demonstrating their potential as reagents for the diagnostic detection of P-glycoprotein in fixed cells and tissues. In Fig. 3C, flow cytometry of cells stained with the monoclonal antibodies showed that the antibody binding correlated with the degree of

drug resistance of the cells, much like the specific P-glycoprotein staining in Fig. 3A. Thus, the staining of permeabilized cells appeared to be due to specific binding of monoclonal antibodies to epitopes of P-glycoprotein exposed on the cytoplasmic aspect of the plasma membrane.

The group I monoclonal antibody C219 has been used to obtain a 600-base pair (bp) cDNA probe, λCHP1, from a λgt11 expression library of the multidrug-resistant CHO cell line, CH^RB30 (see accompanying paper¹⁷). The *lacZ* fusion product of λCHP1, which expresses a C-terminal fragment of CHO P-glycoprotein, is recognized not only by the monoclonal antibody C219, but by all eight antibodies described here¹⁷. Localization of three independent epitopes to a peptide not more than 200 amino acids long was a surprising result as this represents only ~15% of the P-glycoprotein polypeptide. This observation is consistent, however, with the notion that the monoclonal antibodies detect a cytoplasmic domain in fixed cells, as the C-terminus of a transmembrane glycoprotein is often situated on the cytoplasmic side of the membrane. The observation that the monoclonal antibodies bind a fusion protein produced in bacterial cells further confirms that they are directed against the polypeptide moiety of the P-glycoprotein.

The clustering of all three epitopes in the C-terminus of

P-glycoprotein suggests that this region is relatively more immunogenic than most of the protein. The C-terminus possibly is part of a hydrophilic domain in an otherwise relatively hydrophobic protein. Alternatively, it may be a region of the polypeptide that is conformationally more fluid and, therefore, is more likely to be antigenic¹⁸⁻²⁰. It is significant that all eight monoclonal antibodies showed specificity for the same plasma membrane component, supporting the notion that the expression of P-glycoprotein is the major alteration of the multidrug-resistant cell surface. One of the epitopes described here (identified by group I antibodies) seems to be highly conserved among species, but the other two appear to be expressed in a species-dependent manner, as seen in Fig. 1. The latter epitopes may represent more variable regions of P-glycoprotein structure.

The monoclonal antibodies described here will serve as sensitive and specific reagents for the detection of P-glycoprotein in experimental systems and in normal and neoplastic tissues. Furthermore, they have potential for use in screening human tumour biopsies, where early detection of elevated levels of P-glycoprotein might indicate the need for a departure from routine chemotherapy and the application of novel treatments to circumvent the multidrug resistance phenotype^{7,21,22}. Preliminary findings of elevated amounts of P-glycoprotein in some ovarian carcinomas of patients failing to respond to chemotherapy have already demonstrated the usefulness of the present monoclonal antibodies for this purpose¹⁶.

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Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer

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The autocrine hypothesis proposes that a cell produces and secretes a hormone-like substance that can interact with specific membrane receptors on its surface to induce effects such as proliferation¹. Thus, a cancer cell could act to stimulate its own growth. Bombesin and bombesin-like peptides (BLPs) such as gastrin-releasing peptide (GRP) cause various physiological responses in mammals², including stimulation of proliferation of 3T3 mouse fibroblasts³ and normal human bronchial epithelial cells *in vitro*⁴ and induction of gastrin cell hyperplasia and increased pancreatic DNA content *in vivo* in rats^{5,6}. Human small-cell lung cancer (SCLC) cell lines produce and secrete BLPs⁷⁻¹² and can express a single class of high-affinity receptors for BLPs¹³. Exogenously added BLPs can also stimulate the clonal growth and DNA synthesis of SCLC *in vitro*^{14,15}. These findings suggest that BLPs function as autocrine growth factors for this tumour. One way to test this hypothesis is to interrupt the function of the endogenously produced BLPs. Here, we demonstrate that a monoclonal antibody to bombesin binds to the C-terminal region of BLPs, blocks the binding of the hormone to cellular receptors and inhibits the clonal growth of SCLC *in vitro* and the growth of SCLC xenografts *in vivo*. These results demonstrate that BLPs can function as autocrine growth factors for human SCLC.

The C-terminal region of bombesin and GRP, which competes effectively for the same class of high-affinity receptors on pituitary cells and SCLC membranes, is required for receptor binding and physiological activity^{13,16,17}. Murine monoclonal antibodies were prepared against a synthetic analogue of amphibian bombesin (Lys 3-bombesin) which has the same C-terminal heptapeptide sequence as human and porcine GRP^{18,19}. The neuropeptide substance P has the same carboxy-terminal Leu-Met dipeptide as bombesin but is rarely found in SCLC and binds to a different set of receptors than bombesin^{7,16}. Thus, we screened for monoclonal antibodies that bound to bombesin but not substance P. One anti-bombesin monoclonal antibody, 2A11 (IgG₁ κ), showed no cross-reactivity with substance P and was purified from ascites for study.

Using solid-phase synthetic peptides as targets in a radioimmunoassay (RIA), the 2A11 antibody bound to bombesin, the Lys 3 derivative of bombesin used for immunization, the Tyr 4 derivative of bombesin used for iodination, GRP 1-27, GRP 14-27 and GRP 20-27, whereas GRP 22-27, GRP 1-16, substance P and other tachykinins²⁰ showed insignificant 2A11 binding despite some C-terminal homology with bombesin and GRP (Table 1). In addition, a series of unrelated peptides that can be produced by SCLC, such as calcitonin, adrenocorticotrophic hormone (ACTH), arginine vasopressin (AVP) and neurotensin did not bind 2A11. Note that, although SCLCs make BLPs, the 2A11 antibody did not bind to live, intact SCLC (Table 1).

We next used 2A11 to develop a quantitative, competitive RIA system for BLPs using solid-phase 2A11 and ¹²⁵I-labelled Tyr 4-bombesin. This assay was able to detect BLPs in the range of 5-100 pg per well and BLPs were detected by the RIA in extracts of all 10 SCLC lines tested (Table 2). These values are in good agreement with previously reported quantitative data on many of the same tumour lines using rabbit anti-bombesin heteroantisera⁷. In contrast, none of the non-SCLC lines

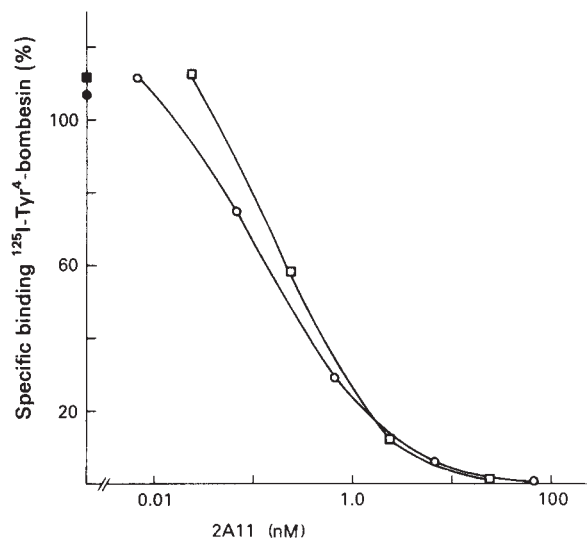


Fig. 1 Inhibition of binding of labelled bombesin in a radioreceptor assay by monoclonal antibody 2A11 using rat brain membranes ($\square$)¹⁶ or small-cell lung cancer (NCI-H345) cell ($\circ$) preparations¹³. The black symbols on the ordinate show that 67 nM MOPC 21 control immunoglobulin did not inhibit the binding of the labelled peptide to either receptor preparation.

Methods. ^{125}I -Tyr 4-bombesin ($50\text{--}80\ \mu\text{Ci}\ \mu\text{g}^{-1}$, 40,000 c.p.m. per test) was incubated with the indicated concentrations of purified monoclonal antibody 2A11 for 60 min at 25° . Receptor binding assays were conducted using 10 mg wet weight of crude rat brain homogenate or 2×10^6 cell of SCLC line NCI-H345 as described previously^{16,30}. The difference in binding in the absence and presence of an excess unlabelled bombesin ($1\ \mu\text{M}$) was taken as a measure of specific binding.

(adenocarcinoma, large-cell and squamous cell lung carcinoma) or the non-lung cell lines (B-lymphoblastoid, T-cell lymphoma and melanoma) expressed detectable levels of BLPs ($<0.01\ \text{pmol}\ \text{mg}^{-1}$ acid soluble protein) (Table 2).

As 2A11 bound to the C-terminal region of BLPs (such as GRP 14-27 and GRP 20-27), we tested the ability of the antibody to block the binding of labelled bombesin to its cell-surface receptor on small-cell lung cancer and rat brain membranes (Fig. 1). Antibody 2A11 blocked bombesin-receptor interaction in a dose-related fashion with both receptor preparations (Fig. 1). Almost complete inhibition of bombesin binding (using $12\text{--}18\ \text{nM}$ labelled bombesin) occurred with $10\ \text{nM}$ 2A11. An indifferent mouse myeloma protein, MOPC 21, of the same isotype as 2A11 ($\text{IgG}_1\ \kappa$) at $67\ \text{nM}$ did not significantly affect bombesin-receptor interaction ($<1\%$ inhibition).

Antibody 2A11 was then examined for its effect on the *in vitro* growth of SCLC cell lines using an agarose cloning assay. Two types of SCLC cell lines were tested against various concentrations of 2A11. The first was a 'classic' SCLC line (NCI-N592) with typical SCLC morphology (growth in tight balls in suspension, scant cytoplasm) and high specific activities of L-dopa decarboxylase²¹. The second was a 'variant' SCLC line (NCI-N417) that deviates morphologically (growth as loose chains, with large cell cytology and abundant cytoplasm) from typical SCLC, has undetectable levels of L-dopa decarboxylase, grows rapidly, clones with high efficiency and is amplified for the *c-myc* oncogene^{21,22}. Both cell lines produce detectable levels of bombesin (Table 2) and both have the SCLC features of high specific activities of the BB isozyme of creatine kinase and neurone-specific enolase^{21,23}. The clonogenic growth of both cell lines was markedly inhibited by $\geq 3\ \mu\text{g}\ \text{ml}^{-1}$ 2A11 (Table 3). In contrast, $10\ \mu\text{g}\ \text{ml}^{-1}$ of the isotype-matched mouse myeloma protein MOPC 21 or boiled 2A11 showed no significant inhibition of SCLC growth in the same assay (Table 3). Exogenously added synthetic bombesin can stimulate the clonal growth of SCLC in serum-free medium (Table 3)¹⁴. The addition of $50\ \text{nM}$ of

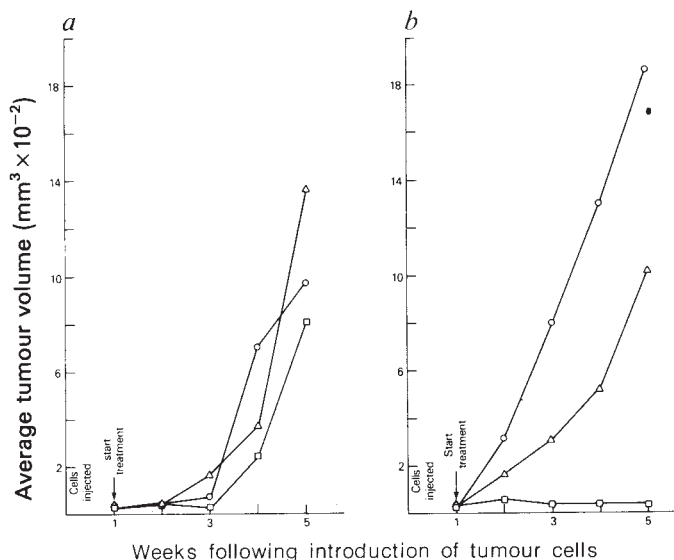


Fig. 2 Effect of treatment with monoclonal antibody 2A11 ($\square$), control monoclonal antibody MOPC 21 (Δ) or PBS ($\circ$) on growth of human tumour xenografts in athymic nude mice. *a*, Human malignant melanoma cell line NCI-H234A; *b*, human small-cell lung cancer line NCI-N592.

Methods. Tumour cells (10^7 /mouse) were subcutaneously injected into the flanks of athymic nude mice (BALB/c *nu/nu*) and a palpable mass formed after 7 days. Treatment begun on day 7 consisted of three trial groups (5 mice per group), each given $0.5\ \text{ml}$ i.p. injections three times a week of either PBS ($\circ$), $200\ \mu\text{g}$ purified MOPC 21 immunoglobulin (Δ) or $200\ \mu\text{g}$ of purified 2A11 antibody ($\square$). Thus, each mouse in the immunoglobulin-treated groups received a total of $600\ \mu\text{g}$ immunoglobulin per week for four weeks. Tumours were measured once a week. Tumour growth is reported as an average relative tumour volume, s.e.m. $\pm 10\%$.

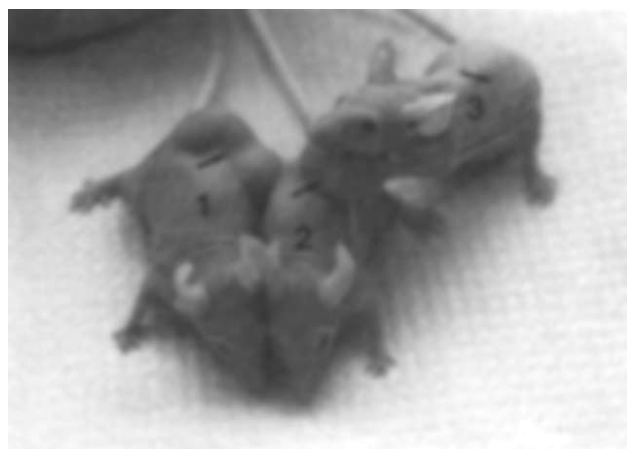


Fig. 3 Representative nude mice 5 weeks after xenografting with small-cell lung cancer NCI-N592 and treated with MOPC 21 (mouse 1), PBS (mouse 2) or 2A11 (mouse 3). Arrows point to the site of tumour inoculation. Large tumours are seen for the two controls (1 and 2) and no tumour mass was detected in mouse 3 at week 5.

exogenous synthetic bombesin added simultaneously with 2A11 completely prevented the 2A11 growth inhibition demonstrating the specificity of the 2A11 effect (Table 3).

Nude mouse heterotransplant studies were used to investigate the ability of 2A11 to inhibit SCLC growth *in vivo*. Two tumour cell lines were used in this study, the classic SCLC line NCI-N592 and a melanoma cell line, NCI-H234A, which did not make BLP and which was unresponsive to bombesin *in vitro*¹⁴, to serve as a control. One week after their inoculation into nude

Table 1 Immunological cross-reactivity of solid-phase synthetic peptides with bombesin using the 2A11 binding assay

Peptide target	Cross-reactivity (%)
Bombesin-like peptides	
Bombesin (BN)	100
Lys 3-BN	98
Tyr 4-BN	96
Gastrin-releasing peptide (GRP 1-27)	102
GRP 14-27	102
GRP 20-27	112
GRP 1-16	0.26
GRP 22-27	<0.02
Tachykinins	
Substance P, physalaemin, kassinin, eliodisin	all <0.02
Unrelated peptides	
Calcitonin, vasoactive intestinal peptide, motilin, β -endorphin, leu-enkephalin, glucagon, somatostatin, neurotensin, xenopsin, ACTH, AVP	all <0.07
Bovine serum albumin (BSA)	<0.02
Live small-cell lung cancer cells	<0.02

BALB/c mice were immunized subcutaneously three times with Lys 3-bombesin conjugated to BSA²⁴ (100 μ g bombesin equivalents per injection; the first dose was in complete Freund's adjuvant, the last two in incomplete Freund's). Monoclonal antibodies were derived by standard methods following fusion to the mouse myeloma cell line X63-Ag8.653 (refs 25, 26). Hybrids were screened for anti-bombesin activity 10 days after fusion by indirect RIA using radio-autography on solid-phase target plates coated with bombesin, substance P or 1% BSA. Hybridomas producing antibodies binding to bombesin but not BSA or substance P were cloned several times. Monoclonal antibody 2A11 (IgG₁ κ) was grown in ascites, purified²⁷ and used for all subsequent tests. For solid-phase RIA, peptides (all purchased from Peninsula Laboratories) were adsorbed to 96-well polyvinyl chloride microtitre plates (Dynatech) by adding 0.05 ml 10 μ g ml⁻¹ peptide in PBS for 1 h, washing in PBS, then blocking with a 1% BSA/PBS solution. Porcine sequence synthetic GRP peptides were used¹⁸. Viable SCLC cell lines NCI-H128, NCI-H209 and NCI-H345 at 10⁵ cells per well were tested in binding assays using 96-well plates with glass filters (V & P Scientific, San Diego)²⁸. Purified 2A11 (10 μ g ml⁻¹) was added, incubated and washed three times. Protein A affinity purified rabbit anti-mouse IgG (Cappel Laboratories) (1.6 μ g ml⁻¹) was added, incubated, washed three times, then ¹²⁵I-protein A (Pharmacia, labelled by chloramine T method to 40–50 μ Ci μ g⁻¹) 40,000 c.p.m. per well was added, incubated, washed eight times and the wells cut out and counted in a Beckman γ -counter. All incubations were for 1 h at room temperature in PBS with 1% BSA in 0.025 ml volumes. Percentage cross-reactivity = (c.p.m. ¹²⁵I-protein A bound on peptide target – c.p.m. bound on BSA target)/(c.p.m. bound by bombesin – BSA background $\times$ 100). The bombesin target caused 2A11 binding sufficient to bind 33,000 c.p.m. of ¹²⁵I-protein A whereas the BSA background bound 30 c.p.m. Mouse myeloma protein MOPC 21 (IgG₁ κ , Litton Bionetics) was used as a control at 10 μ g ml⁻¹ and did not bind to the peptides. All values are the average of triplicate determination and variation between replicates was <5%.

mice each cell line produced a palpable tumour mass. The established tumours were then subjected to three experimental treatments (five mice each group) consisting of: (1) phosphate-buffered saline (PBS); (2) the isotype-matched immunoglobulin MOPC 21; and (3) 2A11. Treatment was administered by intraperitoneal (i.p.) injection three times weekly and tumour growth was monitored as a function of tumour volume over the next four weeks of therapy (Fig. 2). The melanoma cell line, NCI-H234A, showed unhampered tumour growth in all three experimental groups with each group of mice attaining an average tumour volume of 1,000 mm³ by week 5 (Fig. 2a). In contrast, dramatic suppression of SCLC growth (NCI-N592) was observed following 2A11 treatment (tumour mass in all mice <50 mm³) while control groups of MOPC 21- and PBS-treated mice demonstrated rapid tumour growth (tumour mass in all mice >1,000 mm³ by five weeks) (Figs 2b; 3). Note that in MOPC 21-treated controls there was an unexplained tem-

Table 2 Quantitation of BLPs in human cell lines using a competitive RIA with monoclonal antibody 2A11

Cell line providing extract	Immunoreactive BLP (pmol per mg acid-soluble protein)
Small-cell lung cancer lines	
NCI-H128	18.4
NCI-H209	17.4
NCI-H345	7.8
NCI-H510	5.4
NCI-N592	3.9
NCI-H187	3.7
NCI-H69	2.3
NCI-H146	1.6
NCI-N417	0.95
NCI-H446	0.13
NCI-H82	0.04
Non-small-cell lung cancer lines	
A549 (adenocarcinoma)	<0.01
NCI-H23 (adenocarcinoma)	<0.01
NCI-H125 (adenocarcinoma)	<0.01
NCI-H157 (large cell lung cancer)	<0.01
9812 (large cell lung cancer)	<0.01
U1752 (squamous cell lung cancer)	<0.01
Other human cell lines	
NCI-H128BL (B-lymphoblastoid)	<0.01
HCl-H78 (T-cell lymphoma)	<0.01
NCI-H234A (malignant melanoma)	<0.01

Cell lines were grown as described previously^{21,23} and for preparation of cell extracts for RIA, 1–5 $\times$ 10⁷ cells were washed three times in PBS and resuspended in 1 ml of 2 M acetic acid, homogenized, heated in a boiling water bath for 15 min, clarified by centrifugation at 3,000 r.p.m. and the supernatant lyophilized and stored at –80°. The freeze-dried extract was resuspended in 0.5–1 ml PBS, reclarified and protein concentration determined using a BioRad assay kit. Dilutions of this extract were tested in a quantitative competitive RIA. Antibody 2A11 was adsorbed to 96-well polyvinylchloride plates at 500 ng per well followed by saturation with 1% BSA–PBS. Unlabelled bombesin (covering a range of 1–1,000 pg per well bombesin for a standard curve) or cell extract peptides at different dilutions were added to triplicate wells and incubated for 1 h at 25 °C. Without removing the unlabelled peptides, 12,000 c.p.m. of ¹²⁵I-Tyr 4-bombesin (labelled by the chloramine-T method to 50–80 μ Ci μ g⁻¹) was added to each well and incubated overnight at 4 °C, washed eight times and counted. All additions were in 1% BSA–PBS in 0.025-ml volume, and the washes were with 1% BSA–PBS. GRP 1-27, GRP 14-27 and GRP 20-27 were equipotent with bombesin in competing for labelled bombesin and the midpoint of the assay curve detected 25 pg bombesin per well. In contrast, substance P, unrelated peptides and synthetic analogues of bombesin or GRP with substitutions blocking binding to the cellular BLP receptor were unable to significantly compete with labelled bombesin in this assay. In addition, MOPC 21 immunoglobulin did not bind the labelled peptide. Using the standard curve, the BLP equivalent of the cell extract peptides was determined and expressed as pmol of BLP mg⁻¹ of acid soluble protein in the extract. Results are the average of triplicate determinations with variation between replicates of <5%.

porary delay in tumour growth in some mice, but in all cases a large tumour had formed by five weeks.

In the 2A11-treated group, three more weeks of treatment were given; two mice never showed tumour growth, but three developed progressively growing NCI-N592 tumours despite 2A11 treatment. These antibody-resistant tumours are under study. At week 5, the 5 mice with large NCI-N592 tumours in the PBS-treated group were then treated with 2A11 antibody for three weeks and the tumours in all 5 mice stopped growing and became necrotic.

In conclusion, we have reported on a monoclonal antibody to amphibian bombesin that cross-reacts with BLPs of human origin. The antigenic determinant for this antibody has been localized at the C-terminal region of bombesin and GRP. We have demonstrated that 2A11 can be used in an RIA for the quantitative detection of BLPs in extracts of human SCLC. The antibody was shown to block bombesin–receptor interaction in

Table 3 The effect of monoclonal antibody 2A11 on the *in vitro* clonal growth of human small-cell lung cancer lines

Addition to cloning medium	Cell line	
	NCI-N592	NCI-N417
Growth as % control (no of colonies)		
Experiment 1 ($\mu\text{g ml}^{-1}$)		
None	100 (30)	100 (583)
2A11 0.1	93 (28)	98 (571)
2A11 1.0	83 (25)	96 (560)
2A11 3.0	0 (0)	3 (18)
2A11 10	0 (0)	1 (7)
MOPC 21 10	97 (29)	99 (577)
Experiment 2		
None	100 (32)	100 (610)
50 nM bombesin	>100 (664)	>100 (820)
2A11 10 $\mu\text{g ml}^{-1}$	0 (0)	5 (30)
MOPC 21 10 $\mu\text{g ml}^{-1}$	94 (30)	100 (607)
Boiled 2A11 10 $\mu\text{g ml}^{-1}$	88 (28)	97 (593)
2A11 10 $\mu\text{g ml}^{-1}$ + bombesin 50 nM	>100 (340)	>100 (910)

Tumour cell cloning assays were performed in serum-free HITES medium as described previously²⁹. HITES medium contains RPMI-1640 (GIBCO) supplemented with 10 nM hydrocortisone, 5 $\mu\text{g ml}^{-1}$ bovine insulin, 10 $\mu\text{g ml}^{-1}$ human transferrin, 10 nM 17 β -estradiol and 30 nM selenium. A single-cell suspension of NCI-N592 or NCI-N417 cells taken from log-phase cultures was mixed with 0.3% agarose in HITES (v/v) in the absence or presence of purified 2A11 or MOPC 21 antibody, bombesin, boiled antibody or 2A11 + bombesin (added simultaneously) and plated over a pre-hardened base layer of 0.5% agarose/HITES. The NCI-N417 culture plates were supplemented with 0.1% BSA which greatly enhanced clonal cell growth. NCI-N592 cells were seeded at 5×10^4 per plate, whereas NCI-N417 cells were plated at 1×10^4 . Plates were pre-screened to verify single-cell distribution of test cells and scored 21 days later for colony formation. Cell aggregates of >50 cells were scored positive for colony growth. All studies were done in triplicate and each point represents the mean colony count. For all studies the s.e.m. was $\pm 10\%$.

both brain and SCLC membrane preparations. Finally, purified antibody when added to an *in vitro* tumour cloning assay or to an *in vivo* nude mouse xenograft assay prevented tumour cell growth. The antibody effect *in vitro* was blocked by synthetic bombesin, and the antibody did not inhibit the growth of a melanoma tumour cell line that did not make BLPs. We conclude that BLPs can indeed function as autocrine growth factor(s) for human SCLC. It is possible that the bombesin stimulation of growth seen in SCLC could reflect a physiological role of BLPs in some cells during normal fetal growth and development. In addition, our studies with monoclonal antibody 2A11 against BLP offer a new immuno-hormonal approach of manipulating the growth of human SCLC in patients. Although these studies used monoclonal anti-peptide antibodies to inhibit BLP action, it is possible that growth suppressive effects could be obtained using bombesin antagonists or anti-receptor antibodies.

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Translocation of the p53 gene in t(15;17) in acute promyelocytic leukaemia

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Recent studies have demonstrated that the cellular tumour antigen p53 (ref. 1) can complement activated *ras* genes in the transformation of rat fibroblasts, suggesting that the gene encoding p53 may act as an oncogene^{2,3}. Here, by using *in situ* chromosomal hybridization^{4,5}, we have mapped the p53 gene to human chromosome 17, at bands 17q21-q22, the region containing one of the breakpoints in the translocation t(15;17) (q22;q21) associated with acute promyelocytic leukaemia (APL)^{6,7}. Hybridization of p53 and *erb-A* (17q11-q12) probes to malignant cells from three APL patients indicated that the p53 gene is translocated to chromosome 15 (15q+), whereas *erb-A* remains on chromosome 17. Analysis of variant translocations demonstrates that the 15q+ chromosome contains the conserved junction⁸, suggesting a role for p53 in the pathogenesis of APL. However, rearrangements of the p53 gene were not detected on Southern blotting of DNA from leukaemic cells of four APL patients with t(15;17).

To determine the chromosomal localization of the p53 gene, we performed *in situ* chromosomal hybridization using a p53-specific probe. We used an ³H-labelled complementary DNA clone, pp53-176, containing the entire coding region (1.35 kilobases, kb) of the murine p53 gene⁹, for *in situ* hybridization to normal human metaphase chromosomes prepared from phytohaemagglutinin-stimulated peripheral blood lymphocytes. Figure 1 shows the distribution of labelled sites in 100 metaphase cells examined. This hybridization resulted in labelling of a specific region of a single chromosome, namely, the long arm of chromosome 17. Of 100 metaphase cells examined, 24 (24%) were labelled on region q2, bands q21-q25, of one or both chromosomes 17. A total of 183 grains was observed; thus, the labelled sites on region q2 of chromosome 17 represented 14.2% of all labelled sites (26/183). The largest number of grains was clustered at 17q21-q22 (18/183, 10%, $P < 0.005$).

The localization of the p53 gene to 17q21-q22 is of particular interest because of the nonrandom abnormalities involving this

Table 1 *In situ* hybridization of a p53 probe to metaphase cells with a t(15;17)

Patient	No. of metaphase cells analysed	Total no. of labelled sites	No. of labelled sites (%)			
			Normal 15	15 q+	Normal 17	17 q-
1	100	141	5 (3.5%)	14 (9.9%)*	10 (7.1%)†	2 (1.4%)
2	50	73	1 (1.4%)	7 (9.6%)*	7 (9.6%)*	0 (—)
3	63	84	2 (2.4%)	7 (8.3%)†	10 (12%)*	1 (1.2%)

* χ^2 values correspond to $P < 0.05$.

† χ^2 values correspond to $P < 0.20$; however, all grains were clustered on the translocated material from chromosome 17, at bands q21–q22.

chromosome in human tumours. Gain or loss of a whole chromosome 17 has been a frequent finding in haematological malignant diseases as well as in various solid tumours, and structural rearrangements involving 17q are especially frequent in the leukaemias¹⁰. In particular, a reciprocal translocation involving chromosomes 15 and 17 at bands q22 and q21, respectively, is a characteristic abnormality in APL. We have observed this translocation in each of 42 patients with APL that we studied in our laboratory (ref. 7 and our unpublished data).

With regard to the t(15;17), the proto-oncogene *erb-A* has also been mapped to the long arm of chromosome 17. Analysis of hybridizations of an *erb-A*-specific probe to metaphase cells with a t(15;17)¹¹, and Southern blot analysis of somatic cell hybrids retaining the 15q+ chromosome¹², showed that *erb-A* remains on the rearranged chromosome 17. Moreover, no rearrangements of this gene were detected on digestion with eight restriction enzymes of genomic DNAs from five APL patients¹³.

To determine whether the p53 gene or *erb-A* is localized near the chromosomal breakpoint on chromosome 17 that is involved in the t(15;17), we hybridized the two probes to metaphase cells from three APL patients who had this rearrangement. Figure 2 shows the distribution of labelled sites, using each probe, on the rearranged chromosomes and on the normal homologues in cells from patient no. 1. Of 100 metaphase cells examined after hybridization with the p53 probe, 10 (10%) were labelled on the normal chromosome 17; these sites were clustered at 17q21–q22, and represented 7.1% (10/141) of all labelled sites ($P < 0.05$). Two labelled sites were observed on the 17q- chromosome. In 14 metaphase cells, labelled sites were observed on the 15q+ chromosome; they were clustered at bands q21–q22 of the translocated chromosome 17 (14/141, 9.9%, $P < 0.05$). The chromosome 15 homologue had only five labelled sites dispersed along the long arm (3.5%, $P > 0.05$). Thus, the p53 gene, which is normally localized at 17q21–q22, is translocated to chromosome 15 as a result of the t(15;17). Analysis of cells from the two other patients with this rearrangement gave similar results (Table 1).

In contrast, an analysis of metaphase cells hybridized to *erb-A* revealed that this gene remains on the 17q- chromosome and thus is located proximal to the t(15;17) breakpoint (Fig. 2). In 100 metaphase cells examined from patient no. 1, 11 and 10 labelled sites were observed on the normal chromosome 17 and on the 17q- chromosome, respectively (normal 17: 11/125, 8.8%; 17q-: 10/125, 8.0%, $P < 0.05$). In each case, the grains were clustered at 17q11–q12. Two labelled sites were noted on the normal chromosome 15 homologue (1.6%), and four were observed on the 15q+ chromosome (3.2%, $P > 0.05$). Similar results were obtained for hybridizations of metaphase cells from the two other APL patients. This result is consistent with the findings of previous studies in which both *in situ* chromosomal hybridization and Southern blot analysis of DNA from somatic cell hybrids retaining the 15q+ chromosome, were used^{11,12}. Recent results of *in situ* hybridizations to metaphase chromosomes derived from a fibroblast cell line with a different translocation, t(15;17)(q22;q11), suggest that *erb-A* is localized between bands 17q11 and q21 (ref. 13). Our results indicate that the breakpoint on chromosome 17 in the t(15;17) in APL is bracketed by the *erb-A* and p53 genes.

To determine whether the p53 gene is structurally altered by this translocation, we subjected DNA from four APL patients with the t(15;17) to Southern blot analysis using the mouse p53 cDNA probe; human placental DNA served as a germline control. It was necessary to reduce the stringency for these hybridizations, as there is only ~80% homology between the mouse and human genes¹⁴. DNA of high relative molecular mass was prepared and digested with the appropriate restriction enzyme, electrophoresed in 0.8% agarose, and blotted onto Gene Screen Plus transfer membranes (NEN). The blots were hybridized with the plasmid pp53-176, which had been nick-translated with ³²P to a specific activity of 3×10^8 d.p.m. μg^{-1} . Hybridization was for 16 h at 37 °C in 50% formamide, $5 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M trisodium citrate), 10% dextran sulphate, 1% SDS, 20 mM sodium phosphate (pH 6.8), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and denatured salmon sperm DNA at $100 \mu\text{g ml}^{-1}$. After hybridization, the filters were washed in $3 \times \text{SSC}$, 0.1% SDS for 3 h at 55 °C, followed by 30–120 min at 68 °C, as needed, to reduce the background. In all four patients, we observed the following germline bands: *Eco*RI, 18 kb; *Pst*I, 2.9 and 1.6 kb; and *Pvu*II, 4.0 kb. This pattern is consistent with previously published results, except that the 1.6-kb band expected with *Pvu*II¹⁴ was not observed consistently, suggesting that hybridization of this region to the murine probe was weaker than to the human probe. Thus, there were no detectable changes in the structure of the p53 gene in these samples.

Recent studies with the human APL cell line HL60 have demonstrated that most of the p53 gene has been deleted from these cells, except for a small amount of residual 5' sequences¹⁵. From our *in situ* chromosomal hybridizations and Southern analyses, it is clear that there are no major deletions in the p53 gene in fresh APL cells. Our inability to detect new restriction fragments of the p53 gene on Southern blots, however, does not

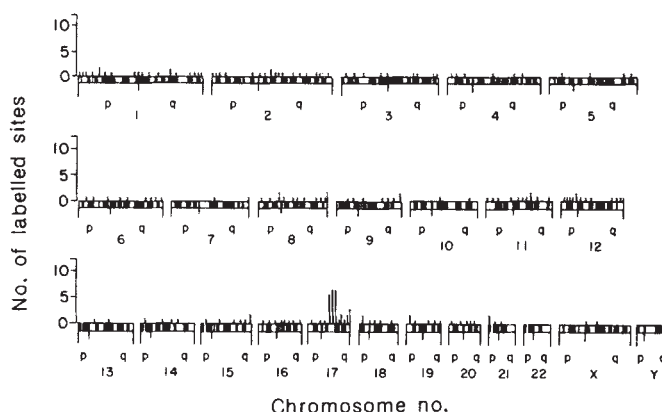


Fig. 1 Distribution of labelled sites in 100 normal metaphase cells. From a total of 29 grains on chromosome 17, 18 (62%) were localized at 17q21–q22. Human metaphase cells prepared from phytohaemagglutinin-stimulated peripheral blood lymphocytes were hybridized with ³H-labelled pp53-176 plasmid DNA nick-translated with all four ³H-labelled deoxynucleotide triphosphates to a specific activity of 2.8×10^7 d.p.m. μg^{-1} . *In situ* hybridization was performed as described previously⁵.

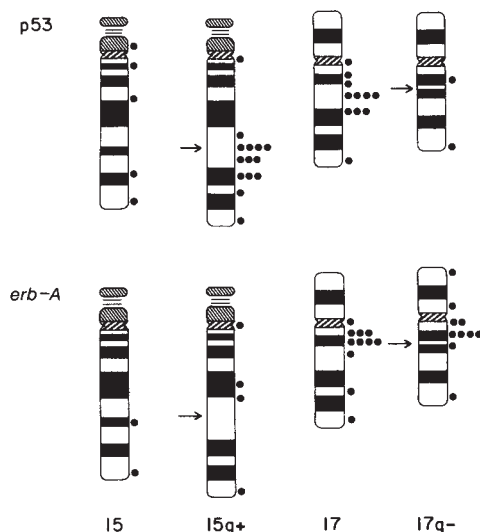


Fig. 2 Distribution of labelled sites on normal chromosome homologues 15 and 17, and on the translocation derivatives 15q+ and 17q-, in metaphase cells from an APL patient (no. 1) with a t(15;17), which were hybridized to the p53 or *erb-A* probes. One hundred metaphase cells were examined for each probe. Arrows identify the breakpoint junctions on the rearranged homologues. The results of these hybridizations indicated that the p53 gene is translocated to chromosome 15, whereas *erb-A* remains on chromosome 17. The radiolabelled probes were nick-translated to specific activities of 2.9×10^7 (p53) and 4.9×10^7 d.p.m. μg^{-1} (*erb-A*). The *erb-A*-specific probe (c-HerbA-1) was a 1.6-kb human *erb-A* cDNA clone¹¹.

exclude the possibility that the gene is activated by the translocation, as a rearrangement may have occurred outside the regions homologous to the probe (protein-coding region). Such a situation has been reported for the *c-abl* proto-oncogene in the t(9;22) associated with chronic myelogenous leukaemia¹⁶. Similarly, in Burkitt's lymphoma, rearrangements are usually seen within the introns of the protein-coding regions of *c-myc* in the common t(8;14); however, such rearrangements occur outside this region in the variant translocations t(2;8) and t(8;22)^{17,18}. Thus, in the latter translocations, rearrangements of *c-myc* cannot be detected with a probe containing only protein-coding sequences.

The location of two genes (the p53 gene and *erb-A*), each with transforming capabilities, adjacent to the t(15;17) breakpoint raises the question as to which gene is involved in the pathogenesis of APL. In a reciprocal translocation such as t(15;17) there are two rearranged junctions, and there is no *a priori* reason for choosing either the 15q+ or the 17q- chromosome as containing the critical junction. The analysis of complex translocations that involve three chromosomes can reveal which of the junctions that are seen in the simple translocation is conserved. Such an analysis has been performed for t(15;17) as well as t(8;14) and t(9;22)⁸; in the latter two translocations, the 14q+ and 22q- (Philadelphia, Ph¹) chromosomes contained the conserved junctions. Subsequent molecular analysis has shown that each of these chromosomes contains the critical genes [*c-myc* and immunoglobulin heavy chain on 14q+ (refs 17, 18), and *c-abl* and *bcr* (breakpoint cluster region) on Ph¹ (refs 16, 19)]. For t(15;17), the 15q+ chromosome junction was conserved in three clearcut complex translocations^{20,21}. In two other reports of variant translocations, however, either the karyotypes permit several interpretations²² or they have not been published²³.

Thus, most of the data available support the notion that the 15q+ chromosome contains the critical junction, and this would suggest that the p53 gene has a critical involvement in the transformation of normal myeloid cells into leukaemic cells whose maturation is arrested at the promyelocyte stage. Further investigation of the role of the p53 gene in t(15;17) awaits

molecular cloning of the junction fragment and analysis of the p53 gene product in these malignant cells.

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Note added in proof: We have recently confirmed the localization of p53 to 17q12-q24 by hybridizing a human p53 cDNA probe to normal metaphase cells. In addition, a smaller yet significant cluster of grains was observed on 17p11-p13; this labelling was not seen with the mouse probe.

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Unusual organization and diversity of T-cell receptor α -chain genes

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T lymphocytes recognize cell-bound antigens in the molecular context of the self major histocompatibility complex (MHC) gene products through the surface T-cell receptor(s). The minimal component of the T-cell receptor is a heterodimer composed of α and β subunits, each of relative molecular mass (M_r) $\sim 45,000$ (refs 1-3). Recently, complementary DNA clones encoding these subunits have been isolated and characterized along with that of a third subunit of unknown function, termed γ (refs 4-9). These studies revealed a primary structure for each subunit that was clearly similar to that of immunoglobulin and indicated a somatic rearrangement of corresponding genes that are also immunoglobulin-like. Recently, the analysis of the sequence organization of the T-cell receptor β -chain¹⁰⁻¹⁶ and T-cell-specific γ -chain gene families¹⁷ has been reported. We now present an initial characterization of the murine T-cell receptor α -chain gene family, and conclude that although it is clearly related to the gene families encoding immunoglobulins¹⁸, T-cell receptor β -chains¹⁰⁻¹⁶ and also T-cell γ -chains¹⁷, it shows unique characteristics. There is only a single constant (C) region gene segment,

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which is an exceptionally large distance (~20–40 kilobases (kb) in the cases studied here) from joining (*J*) gene segments. In addition, the *J* cluster and the variable (*V*) segment number seem to be very large. Finally, in the case studied here, a complete α -chain gene shows no somatic mutation and can be assembled directly from V_α , J_α and C_α segments without inclusion of diversity (D_α) segments.

We reported previously⁶ the isolation of a cDNA (pHDS58) from a cloned cytotoxic T lymphocyte (2C) that encodes the T-cell receptor α -chain¹⁸. The sequence of this cDNA clone showed that, by analogy with immunoglobulin genes, the cDNA can be divided into *V*, *J* and *C* regions⁶. The whole α -cDNA, or its fragments containing one or more regions, was used to probe *Eco*RI-digested BALB/c embryo DNA. The results can be summarized as follows (Fig. 1). First, there are at least four fragments (20, 15, 9 and 2.2 kb) carrying V_{2C} or V_{2C} -like sequences, indicating that the pHDS58 *V* region is derived from a family of closely homologous germline *V* DNA segments. Second, the 2C *V* probe hybridized most intensely with the 2.2-kb fragment, suggesting that this fragment contains the germline counterpart of the pHDS58 *V* region. Third, the pHDS58 *J* region is encoded in an *Eco*RI fragment of ~800 base pairs (bp). Fourth, *Eco*RI fragments of 6.2 and 3.9 kb contain the genetic information for the 3' and 5' ends of the *C* region, respectively, suggesting that, unlike the β - and γ -genes, the BALB/c embryo contains only one *C* gene segment for the α -chain. In addition, when we analysed BALB/c embryo DNA using *V*-region probes derived from several other α -chain cDNAs, we detected different hybridization patterns composed of multiple bands (data not shown), suggesting that, unlike the β -chain gene family, the α -chain gene family contains many germline V_α DNA segments.

The 2.2-kb *Eco*RI fragment containing the germline counterpart of the pHDS58 V_α region was cloned (clone λ 2.2) and sequenced. The sequence is identical to that derived from the corresponding region of pHDS58, indicating that somatic mutation did not occur in the formation of the complete α gene (Fig. 2a). The same conclusion has been reached for a β -chain¹² and for T-cell γ genes¹⁷.

DNA clones containing the various genomic DNA sequences of the J_α - C_α region in the germline configuration are summarized in Fig. 3a (see legend for details of the derivation of these genomic clones). All genomic clones isolated with phage λ vectors are from BALB/c and the cosmid clone 11E is from C57BL/6. However, the Southern blot analysis (Fig. 3b) indicates that there is no gross length polymorphism between these two strains in the relevant region.

We determined the exon-intron structure of the C_α sequence by DNA sequencing (Fig. 2c). Apart from the overall homology with the *C*-gene segments of immunoglobulins, T-cell receptor, β -chain and T-cell-specific γ genes, a striking feature of the α -chain *C* region is its shortness. This is primarily reflected in the first exon which encodes a *C* domain of only 87 amino acids. By contrast, the analogous exons of the T-cell receptor β -chain genes and of the immunoglobulin heavy-chain genes and T-cell γ genes are, respectively, 124 (ref. 10) and 110 amino acids^{17,19}. The second *C*-segment exon is similar to that of the T-cell receptor β -chain and T-cell γ -gene *C* segments in encoding only a short, cysteine-containing peptide of similar size, but not homologous in sequence, to the exon that encodes the hinge region of IgG²⁰. The third exon primarily encodes the putative transmembrane domain and the proposed cytoplasmic tail, and the fourth exon encodes only 3' untranslated region. Terminal exons that encode only untranslated region are a feature of some class II MHC genes²¹, but are without precedent among immunoglobulin, T-cell receptor β -chain^{10,11} and T-cell γ genes¹⁷.

The *J*-segment sequence (Fig. 2b) derived from clone λ 9.1 (Fig. 3) clearly represents the *J* segment used to assemble the α -chain gene (pHDS58) active in 2C cells. It demonstrates two features that are highly conserved among *J* segments: the nucleotide sequence GGNNNGGN that encodes 'G-G',

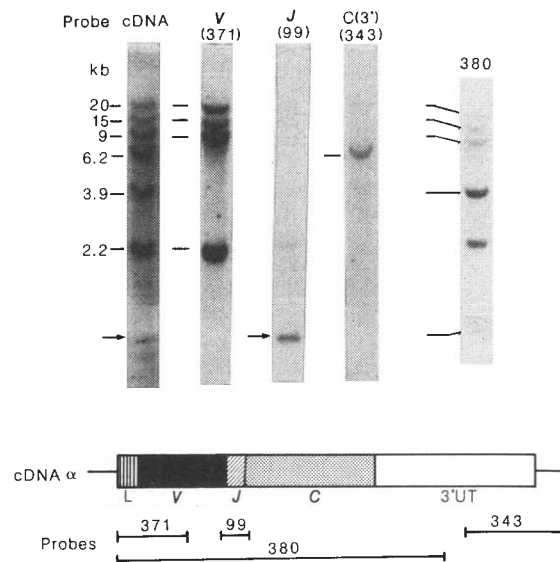


Fig. 1 *Eco*RI-cleaved BALB/c embryo DNA electrophoresed through 0.9% agarose gels, transferred to nitrocellulose (BA85)³², and hybridized to probes derived from cDNA pHDS58 as indicated in the diagram. Probes were labelled by nick translation³³, and the sizes (kb) of the fragments detected are indicated. All samples, except those probed with probe 380, were run on the same gel, which was subsequently divided into strips. The arrow indicates a fragment of ~800 bp, which is detectable with *J* probe (no. 99) and faintly with other *J*-containing probes. Fragment sizes were determined from co-electrophoresis of phage λ DNA cut with *Hind*III and *Sal*I.

which is invariant in functional *J* segments outside the γ -gene family, and the presence 5' to the *J*-coding region of the conserved nonamer and heptamer sequences implicated in *V*-*J* rearrangement (Fig. 4)²². The sequences, separated by 12 bp, are essentially complementary to a heptamer and nonamer that occur immediately 3' to the V_α segment separated by 23 bp (Figs 2a, 4). The spacings of 23 bp in the sequences 3' to V_α and of 12 bp in the sequences 5' to J_α are common among the three T-cell-specific rearranging genes, α , β and γ , and suggest (according to an empirical rule^{23,24}) that these *V* segments can join directly to the respective *J* segments without the involvement of the third (*D*) segment (see below).

By the criteria of the conserved 'G-G' coding triplets and the conserved heptamer and nonamer sequences, we searched for additional *J* segments and found a second (J_1) *J* segment >700 bp downstream of the first one, and a third one (J_2) ~0.5 kb downstream of the second (Figs 2b, 3a). A fourth, J_{C10} used by the α -chain of a helper T-cell hybridoma, was mapped by Southern blotting to ~4.0 kb downstream of J_2 . Three more *J* segments (J_{C11} , J_{B8C3} and J_{DFL12}) were mapped on another genomic clone, λ 8.2, whose DNA occupies a region upstream of the C_α gene segments (Fig. 3a). Thus, we have mapped seven different J_α segments over a distance of ~20 kb. More *J* segments may exist in the 19-kb region between J_{DFL} and C_α , and possibly also 5' to J_{2C} . By comparison, the two T-cell receptor J_β clusters are spread over 1.1 and 1.9 kb (refs 10, 12), the immunoglobulin J_H cluster over 1.36 kb (refs 23, 25) and the immunoglobulin J_κ cluster over 1.4 kb (refs 22, 26). In each of these cases the *J* segments are relatively evenly spaced ~50–500 bp apart. Given that the first seven J_α segments we have identified were all different and that our analysis already points to clustering of some J_α segments (J_{2C} , J_1 and J_2 ; and J_{C11} , J_{B8C3} and J_{DFL}), the α -gene family must contain many more J_α segments. If there are 20 J_α segments, the probability that the first seven analysed would be different is significant (30%). Our analysis of *J* segments shows those used by cytotoxic T lymphocytes and by T-helper cells to be interspersed one with another, as with T-cell receptor β -chain segments^{10,12}, implying

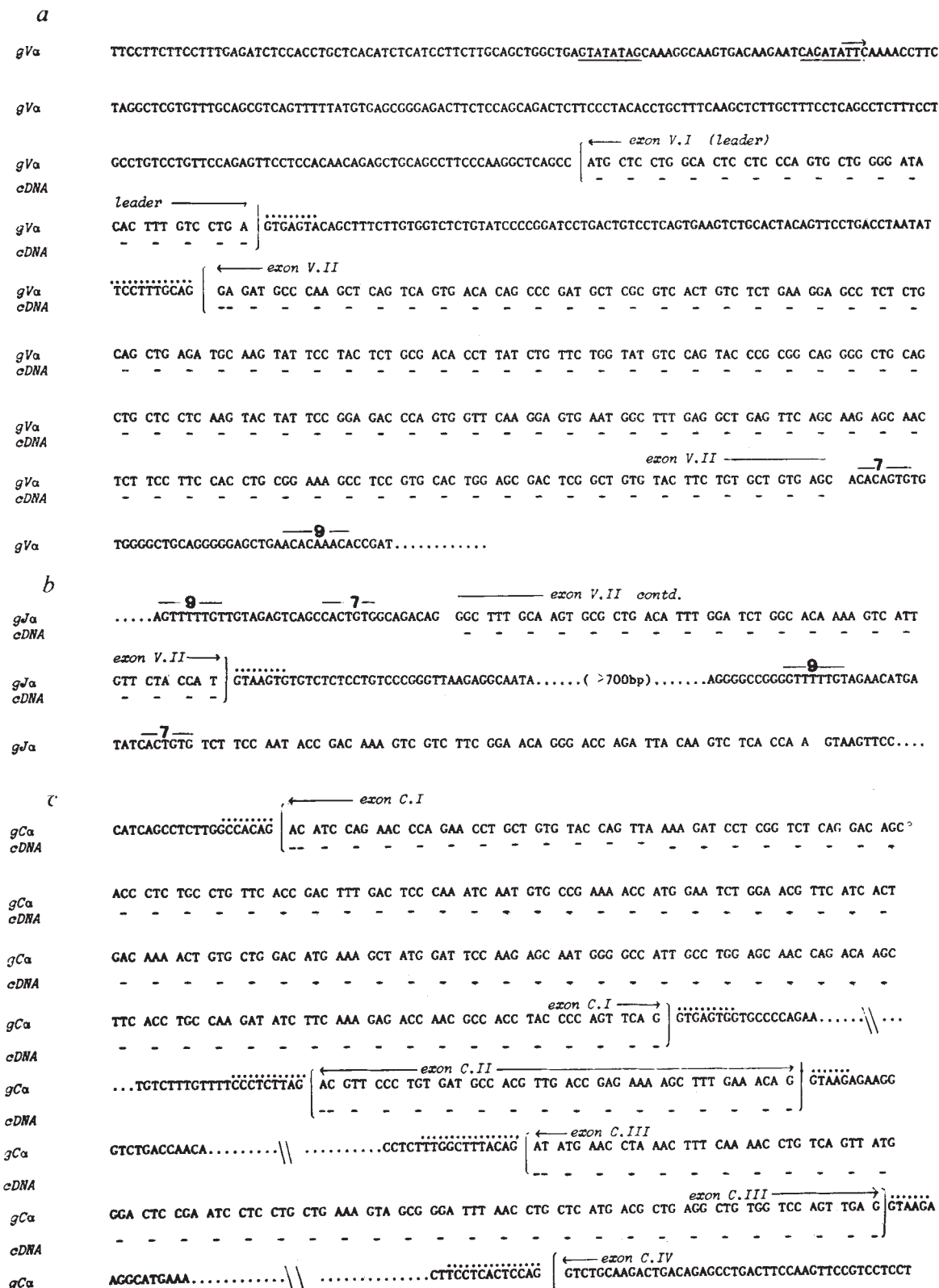


Fig. 2 Comparison of T-cell receptor α -chain sequences and cDNA α -sequences. *a*, Comparison of germline V sequence (gV_{α}) determined using clone λ 2.2 as described in ref. 34 and cDNA pHDS58 sequence⁶ throughout the V segment. The perfect homology of the two sequences is indicated (for the protein coding regions only) by dashes, but extended upstream of that for all the available pHDS58 sequence⁶. A candidate transcriptional start site is superscripted with an arrow, being within a sequence (underlined) resembling Py...PyAPyPyPyPy³⁵. Between 20 and 30 bp upstream of this is a candidate TATA box (underlined)³⁵. The exon V.I denotes the putative leader region although the transcript of this region is contiguous with that of upstream untranslated sequence. The splice donors and receptors are overlined with dots. The proposed heptamer and nonamer V segment signal sequences at the 3' end of V are indicated. *b*, Comparison of germ line J_{α} sequence (gJ_{α}) and pHDS58 sequence through the J segment. The J_{α} segment completes the α -chain V region, that is, exon V.II. 3' of this is shown sequence of another putative J_{α} segment (J_1) from downstream in the germline. *c*, Comparison of germline C_{α} sequence (gC_{α}) and pHDS58 sequence throughout the C segment. Homology is shown by dashes only for the protein coding sequences. Exon C.IV encodes only untranslated RNA. The germline sequence of this continues for over 500 bp with the same sequence as was determined for pHDS58 (data not shown).

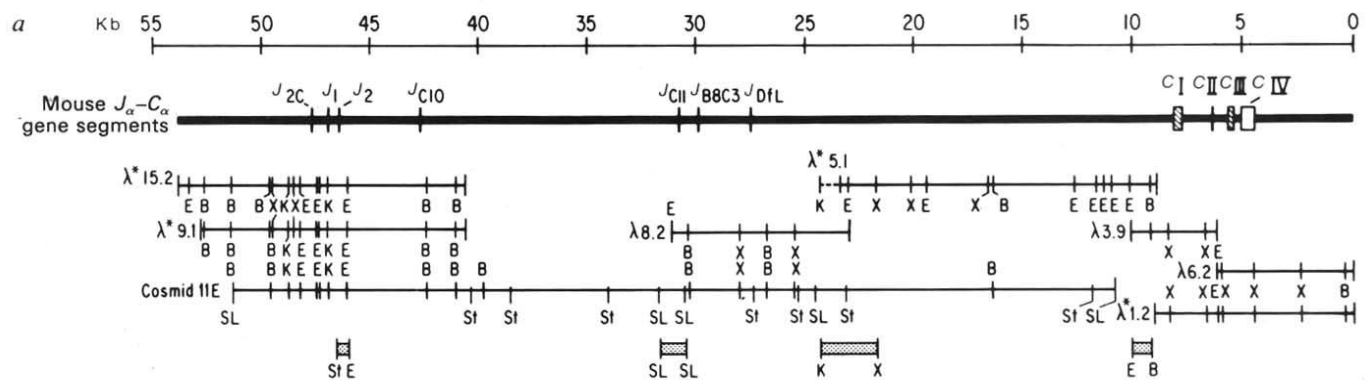
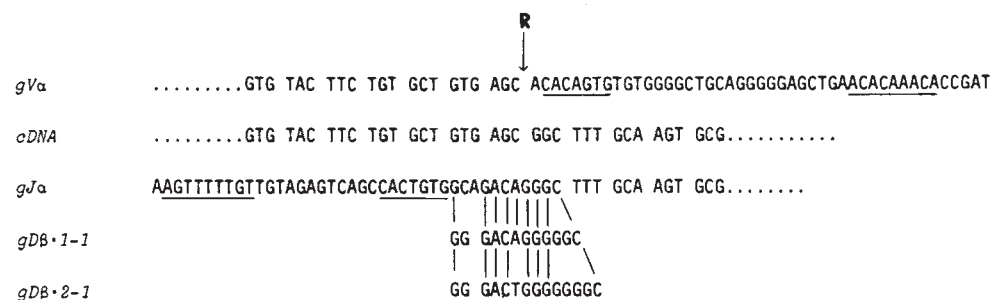


Fig. 3 The murine embryonic *J-C* region. *a*, The map was deduced by analysis of the recombinant clones indicated. Clones λ 3.9 and λ 6.2 were derived from a library of six-fractionated BALB/c embryo *EcoRI* fragments using probes 380 and 343 (see Fig. 1), respectively. Clone λ 8.2 derived from the same library using a *J* probe from a cDNA α derived from T_H hybridoma B8C3 (L. Glimcher and P. Allen, manuscript in preparation). Clones λ *1.2, λ *5.1 were derived from a Charon 4A library of partially *EcoRI**-digested BALB/c embryonic DNA using the 800-bp *EcoRI*/*Bam*HI fragment EB from λ 3.9 as the hybridization probe. Clone λ *15.2 and λ *9.1 were derived from the same library using probe 99 (Fig. 1). Cosmid 11E was derived from a partial *Sau*3A library of C57.B6 embryo DNA. The maps of the clones, assignment of coding sequences and overlap between clones were determined by restriction analysis, cross-hybridization and DNA sequence, and were confirmed by genomic Southern analyses of embryonic DNA of BALB/c and of C57.B6 mice. As an example, the probe KX, stretching from a *Kpn*I site in the L arm of Ch4A to an *Xba*I site in clone λ *5.1, detects in the murine germ line a *Bam*HI fragment of 10.2 kb and an *Xba*I fragment of 3.8 kb, consistent with the mapping shown here. Furthermore, the *Bam*HI/*Eco*RI fragment of λ 8.2 in turn hybridized to KX, as well as to the 400-bp fragment that lies between the left-most *Eco*RI site in the insert of λ *5.1, and the L-arm. Restriction sites are: E (*Eco*RI), B (*Bam*HI), X (*Xba*I), K (*Kpn*I), SL (*Sal*I) and St (*Sst*I). Maps are not necessarily complete, notably for *Eco*RI and *Xba*I on cosmid 11E. In addition to B8C3, *J_α* segments were localized for T_H hybridomas C10 and C11 (Glimcher and Allen, in preparation), and for cytotoxic T-lymphocyte clone DFL.12 (ref. 36). Exons are boxed and shaded if translated. Germline *V_{2Cα}*, cloned in λ V2.2, lies upstream at an as yet undetermined distance from *J_{2C}*. *b*, Southern analysis of BALB/c and C57.B6 genomic DNA using cosmid 11E-derived probes. Embryonic DNA from BALB/c and C57.B6 mice was co-electrophoresed on 1.2% agarose gels after restriction with *Sst* (St) or *Kpn* (K) and *Sal* (SL). Southern transfers of the electrophoresed DNA were probed with either the 1.2-kb *Sal* fragment (dotted line in *a*) for St-cut DNA or the 0.6-kb St-E fragment (dotted line in *a*) in the case of SL-K-cut DNA. Methods are further described in Fig. 1 legend.

Fig. 4 Comparison of germline *V_α*(g*V_α*), germline *J_α*(g*J_α*) and cDNA pHDS58 sequences indicates recombination of *V_α* to *J_α* at point R. The germline g*J_α* has homology at its 5' end to sequences of D β .1-1 and D β .2-1 (refs 13, 14). The proposed heptamer and nonamer sequences 3' to V and 5' to J, respectively, are underlined.



that there is no functional clustering of *J* segments in either gene family.

One consequence of the large *J* cluster is the presence of an unusually long intron between *V*-(*D*)-*J* and *C* segments in at least some assembled α genes. For example, in the 2C α gene this intron is ~40 kb, which is without precedent among immunoglobulin or immunoglobulin-like genes characterized to date. Very large introns have recently been discovered in the *Ubx* and *Antp* loci of *Drosophila melanogaster*²⁷; in *Drosophila*, the large intron may be differentially processed, whereas here the primary function of the large intron may be to accommodate many *J* segments. Because of the similarity between both the gene segments that comprise T-cell receptor and immunoglobulin genes and the overall organization of these gene families, transcriptional activity at the promoter of a rearranged *V_α* gene segment may be enhanced in an analogous manner to that of rearranged immunoglobulin *V_H* and *V_κ* segments. However, if a tissue-specific enhancer (analogous to the immunoglobulin enhancer) does exist in the *J-C* intron of the T-cell receptor α -chain genes, it must be activating the promoter

associated with the rearranged *V_α* gene segment at a distance of at least 20 kb in 2C cells. Alternatively, the existence of such a large *J_α* cluster may have excluded the activation of *V_α* segment transcription by this mechanism.

One interesting feature of the *J_{2C}* segment is its unusual length of 63 bp (Fig. 2b)^{10,12,25}. At its 5' end, the *J* sequence GCAGACAGGGC resembles the sequences of the T-cell receptor β -chain gene *D* segment^{13,14} (Fig. 4). Thus, the *J_{2C}* segment can be regarded as a *D*-type element of 11 bp pre-fused in the germ line to a *J*-type element of 52 bp. In both IgH and T-cell receptor β -gene families, single *D* elements occur in relatively close proximity to the respective *J* clusters^{13,14,28}. It is possible that the analogous *D_α* segment does not exist, having already fused to *J_α* to generate the *J_{2C}* segment in the germ line. The implication is that, albeit at low frequency, cells other than lymphocytes can effect *D-J* recombination.

Another interesting feature of the *J_{2C}* segment is its direct joining with the germline *V_{2C}* segment in 2C cells. The fusion of the germline *V_{2C}* and *J_{2C}* segments should create a novel *V_α* probe reactive *Eco*RI fragment of 1.9 kb in 2C cells (probe 371)

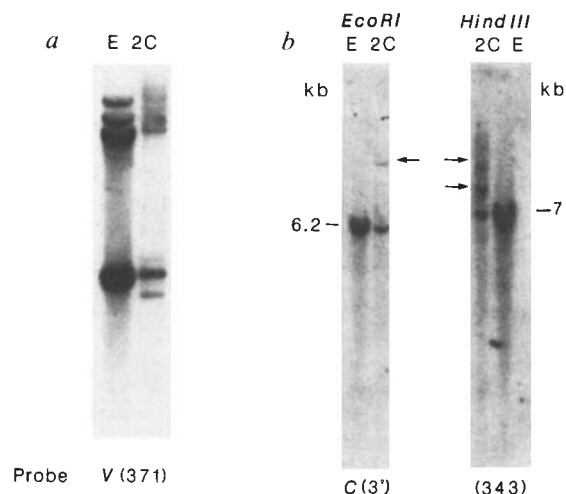


Fig. 5 *a*, *Eco*RI-cleaved DNA of BALB/c embryo, E and cytotoxic T-lymphocyte clone 2C cells, electrophoresed through 0.9% agarose, and analysed as described in Fig. 1. *b*, *Eco*RI-cleaved and *Hind*III-cleaved DNA of BALB/c embryo, E and 2C cells, analysed as described in Fig. 1 and hybridized with probe 343 (see Fig. 1). Bands strongly detected in 2C DNA but not in embryo DNA are arrowed.

that would not be present either in germline DNA, B-cell DNA or DNA of T cells that have not made this particular V-J combination. Such a fragment is detected in 2C DNA (Fig. 5a). In IgH, T-cell receptor β -chain or T-cell γ -gene families, extra nucleotides are commonly found between the fused V and J segments¹⁸. These insertions are either template-independent or the result of the participation of a third type of germline DNA segment, D, in the fusion process^{13,14,28,29}. Although the 12/23-bp spacer rule permits direct V-J joining for the T-cell β -receptor and T-cell γ -gene families, no such case has yet been clearly demonstrated. By contrast, the germline V_{2C} and J_{2C} sequences presented here can fully account for the sequence of pHDS58 in the J region (Fig. 4), strongly suggesting that a direct V-J joining has taken place. However, α -cDNA sequences derived from other cytotoxic T-lymphocyte clones and helper T-cell hybridomas (unpublished data) as well as the sequences of other germline J_{α} segments strongly suggest that complete α genes are also assembled with the participation of D segments.

Probe 343 (Fig. 1) derives solely from the 3' end of C_{α} and detects in BALB/c embryo DNA a single *Eco*RI fragment of 6.2 kb and a single *Hind*III fragment of ~7 kb. Surprisingly, when this probe is directed against *Eco*RI- or *Hind*III-cleaved 2C cell DNA, it detects additional fragments (Fig. 5b) which, because of their reproducibility, are apparently not caused by partial digestion of 2C cell DNA. The nature of the gene rearrangement in 2C cells relative to the BALB/c germ line that generates these novel fragments is unclear. Candidates include chromosomal rearrangements 3' to the C_{α} locus, or amplification of germline sequences which, when in single copy, are undetectable with probe 343. Note that in the experiments shown in Fig. 5b, an excess of digested BALB/c embryo DNA was loaded in an effort to detect in the germline single-copy, unrearranged versions of the novel bands present in 2C cells.

In conclusion, our characterization of murine gene segments encoding the T-cell receptor α -chain indicates a multi-gene family that is comprised of many V-gene segments, a large array of J-gene segments and a single C_{α} gene. Furthermore, our data strongly suggest that a given pair of V_{α} and J_{α} segments can join either directly, without the participation of a D_{α} segment, or indirectly, through one or more D_{α} segment(s). Thus, the scope for combinatorial diversity of the α -gene family seems to be significantly greater than that of IgH, Ig κ and T-cell receptor β - and γ -gene families. By contrast, the data presented here for the 2C α gene and those reported earlier by others for β genes¹²

and by us for γ genes¹⁷ did not give any indication of somatic mutation, an important source of diversity for immunoglobulin genes¹⁸. This apparent lack of somatic mutation in T-cell receptor genes may be related to the relatively large combinatorial diversity seen in the α -gene family, the latter partially compensating for the former. However, apparent somatic mutation of both α - and β -chain T-cell receptor genes was noted on *in vitro* passage and subcloning of an interleukin-2-secreting T-cell hybridoma³⁰. The role of somatic mutation in diversifying the T-cell receptor repertoire³¹ remains an open question.

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Genomic organization of the genes encoding mouse T-cell receptor α -chain

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The vertebrate immune system uses two kinds of antigen-specific receptors, the immunoglobulin molecules of B cells and the antigen receptors of T cells. T-cell receptors are formed by a combination of two different polypeptide chains, α and β (refs 1-3). Three related gene families are expressed in T cells, those encoding the T-cell receptor, α and β , and a third, γ (refs 4-6), whose function is unknown. Each of these polypeptide chains can be divided into variable (V) and constant (C) regions. The V_{β} regions are encoded by V_{β} , diversity (D_{β}) and joining (J_{β}) gene segments that rearrange

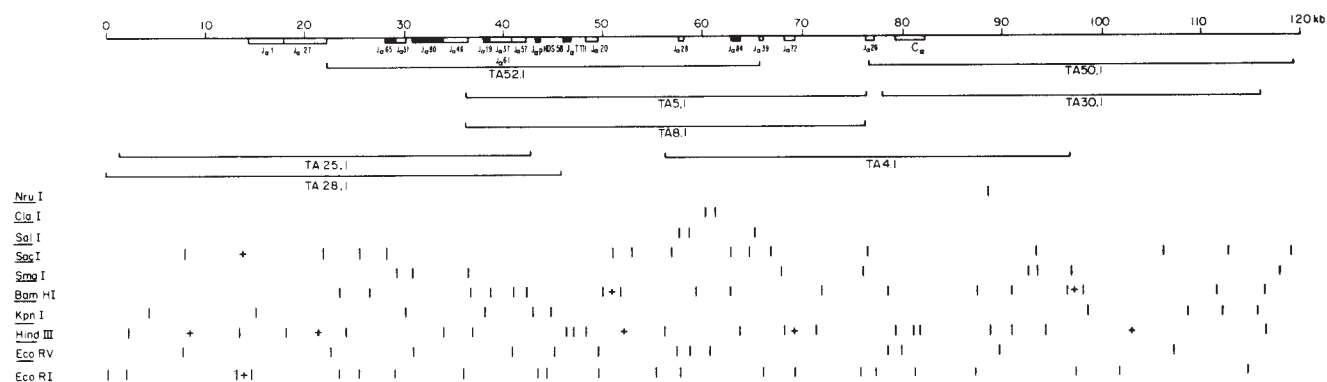


Fig. 1 Restriction map of the mouse cosmid cluster containing the J_α gene segments and the C_α gene. Open boxes indicate restriction fragments that hybridize to the 500-bp *Sau3A* fragment C_α probe from A10 cDNA clone or to the synthetic oligonucleotide J_α probes. Solid boxes indicate the J_α gene segments that have been sequenced in this paper. Unassigned sites between two restriction sites are indicated by +. The J_α gene segments lie to the 5' side of the C_α gene whose direction of transcription is to the right. The J_α gene segments are named according to the cDNA clone from which they originated^{15,16,18}. The cDNA library was constructed in λ gt10 vector as described³⁸, with minor modification. The second-strand synthesis was done using RNase H, DNA polymerase I and T4 ligase³⁹ and the unincorporated nucleotides were removed at every step by NH_4 -acetate/ethanol precipitation. The cosmid library was constructed and screened as described previously⁴⁰. The oligonucleotides for J_α gene segments were purified in a 20% preparative polyacrylamide gel. The DNA band was visualized by placing a silica gel 60F-254 precoated TLC plate (EM reagents) under the gel and shining short ultraviolet light from above. The DNA band was then cut out, crushed and mixed with 3 ml 0.5 M NH_4 -acetate, 10 mM Mg-acetate, 1 mM EDTA, 0.1% SDS and elution was at 37 °C for 3 h with shaking. The acrylamide was then separated from the DNA by spinning in a Quick-sep Isolab column and the aqueous solution containing oligonucleotide was concentrated to <0.2 ml by several *sec*-butanol extractions. Desalting of DNA was done in a 5 ml Sephadex G25 column. Fractions were collected and monitored by a spectrophotometer. The pure oligonucleotides were kinased as described previously⁴¹. Hybridization of the J_α oligonucleotides was done in $6\times\text{SSC}$, $5\times\text{Denhardt's}$, 0.1% SDS and $100\text{ }\mu\text{g ml}^{-1}$ denatured salmon sperm DNA at 37 °C with 5×10^5 c.p.m. ml^{-1} . The blots for oligonucleotides were washed at 25 °C in $6\times\text{SSC}$, 0.1% SDS.

in the differentiating T cell to generate V_β genes⁷⁻¹⁴. The V_γ regions are encoded by V_γ , J_γ and, possibly, D_γ gene segments^{4,5}. Studies of α complementary DNA clones suggest that α -polypeptides have V_α and C_α regions and are encoded by V_α and J_α gene segments and a C_α gene¹⁵⁻¹⁷. Elsewhere in this issue we demonstrate that 18 of 19 J_α sequences examined are distinct¹⁸, indicating that the J_α gene segment repertoire is much larger than those of the immunoglobulin (4-5) or β (14) gene families. Here we report the germline structures of one V_α and six J_α mouse gene segments and demonstrate that the structures of the V_α and J_α gene segments and the α -recognition sequences for DNA rearrangement are similar to those of their immunoglobulin and β -chain counterparts. We also show that the J_α gene-segment organization is strikingly different from that of the other immunoglobulin and rearranging T-cell gene families. Eighteen J_α gene segments map over 60 kilobases (kb) of DNA 5' to the C_α gene.

A full-length cDNA probe, A10, was obtained for the isolation of germline V_α , J_α and C_α clones from a λ gt10 cDNA library of the T-cell helper hybridoma 1.9.2 using an oligonucleotide probe specific for the C_α gene^{15,16}. DNA sequence analysis showed that the A10 clone was identical in sequence to the published full-length cDNA clone TT11 derived from a T-cell hybridoma¹⁵. A V_α -specific probe was derived from the A10 cDNA and used in Southern blot analysis to demonstrate a V_α rearrangement in the BW5147 tumour DNA (data not shown). As both α -sequences come from different T-cell hybridomas which have the BW5147 cell line as a common parent, these observations suggest that clone A10 is derived from the T-cell tumour BW5147 α -gene. This V_α -specific probe was then used to isolate both germline and rearranged clones of the BW5147 V_α subfamily from a λ genomic library of the 1.9.2 hybridoma DNA.

A C_α -specific probe was obtained from the A10 cDNA clone and used in Southern blot analysis of several mouse DNA clones digested with various restriction enzymes. These data indicate that there is only one cross-hybridizing C_α gene (data not shown). The C_α -specific probe was then used to screen a cosmid library constructed from liver DNA of the inbred B10.D2-H-2^{dml} mouse to obtain three overlapping cosmid clones (Fig. 1). Two successive chromosomal walks were then performed with single-copy probes isolated from the 5' ends of cosmid clones TA4.1 and TA52.1 to yield five additional clones (Fig. 1). These eight

cosmid clones encompassed 119 kb of DNA and contained all 18 J_α gene segments described elsewhere in this issue¹⁸.

To study the structure of the V_α gene segment, we isolated a germline V_α clone, V_α 5H, using the V_α -specific probe from the A10 cDNA. The V_α 5H has a leader exon of 49 nucleotides separated by an intron of 188 nucleotides from a V exon of 287 nucleotides (Fig. 2a). The leader coding segment is split by an intron probably four codons from its 3' end. The V_α 5H sequence seems to represent a functional V_α gene segment and belongs to the V_α 1 subfamily which contains at least 10 different members (ref. 18 and A.W., unpublished data). The V_α 5H coding region is 96% homologous at the DNA level and 98% homologous at the protein level to the coding region of the A10 cDNA clone, hence these sequences represent closely related members of the V_α 1 subfamily (see ref. 18).

The 3' end of the V_α gene segment is marked by a recognition sequence for DNA rearrangement¹⁹. It consists of a conserved heptamer sequence CACAGTG, a 21-base pair (bp) spacer sequence and an A+T-rich nonamer sequence (Fig. 2). The recognition signal is very similar to those of the κ and heavy (H) immunoglobulin¹⁹ and β and γ T-cell gene families^{5,7,8,10,11}.

The C_α gene is 79 kb from the 5' end of the cosmid cluster and 37 kb from the 3' end (Fig. 1 and A.W., unpublished data). The locations of J_α gene segments were determined using 18 oligonucleotide probes that were synthesized corresponding to the 5' or 3' ends of the J_α cDNA sequences^{15,16,18}. As the 5' and 3' ends of the J_α coding regions are different from one another¹⁸, the oligonucleotide probes do not cross-hybridize in the conditions used. In each case only one restriction enzyme fragment from the cosmid clones hybridized to each of the individual oligonucleotide probes. Eighteen J_α gene segments map over a region which extends from 3 to 63 kb 5' to the C_α gene (Fig. 1). The precision in the assignment of the J_α gene segment locations varies within 0.5 to 4 kb of DNA because of the limited number of restriction enzymes that we have used to map the cosmid clones. Hence, the distribution of J_α gene segments is strikingly different from the distribution of the J gene segments of immunoglobulin and the T-cell β - and γ -genes. The J_α gene segments are spread over at least 60 kb of DNA, whereas the J_β and J_γ gene clusters extend over only 2 kb of DNA. Assuming that the 18 J_α gene segments analysed are a random representation of the J_α gene segment repertoire, the fact that all of them

a

Leader

Germline V_h 5H 1 M K S L T G A G T V S S L T V G T C L W L C G L T A A A C T G T A A G T T T G G A A T T C C T T T G G G A T C A G G T G T A 93

cDNA V_h AIO 1 A T G A A A T C C T T G A G T G T T C C C T A G T G T C T G T G G C T C L L L N N C T A A C T T G T A A A C T 49

Germline V_h 5H 84 T A G A A A A G T A C T G T C A G C T C C A G G A A A T G T G A G A A T C A C A G A T G T C T T A A T C T C A G T T A A G A A A T G G G A A G C C C A T G G A A A A A T C A T T C T A G A G T G 183

Variable

Germline V_h 5H 184 T T A G C A T C C A T A C G T A T C C A G T C T T G A T T G T C T G A C C T T T G T C T T T C T G C A C A G G G T G A A C A G C C A G C A G A A G G T G C A G C A G A G C 269

cDNA V_h AIO 50 A T G A A A T C C T T G A G T G T T C C C T A G T G T C T G T G G C T C L L L N N C T A A C T T G T A A A C T T G T A A A C T 81

Germline V_h 5H 270 P E S S L I V P E A G G A A M T S L N C T F S D S A S Q Y 344

cDNA V_h AIO 82 C C A G A A T C C T A T T G T C C A G A G G A A A T G A C C T C T C A A C T G C A C T T T C A G T A T 156

Germline V_h 5H 345 F A N Y R Q H S G K A P K A L M S I F S N G E K E 419

cDNA V_h AIO 157 T T C T G G T G G T A C A G A C A T T C T G G A A A G C C C A A G G A C T G A T G T C C A T C T T C C A A T G G T G A A A A A G A A 498

Germline V_h 5H 420 E G R F T I H L N K A S L H F S L H I R D S Q P S 494

cDNA V_h AIO 232 G A A G G C A G A T T C A C A T T C A C C T C A A T A A A G C C A G T C T G C A T T C T C G C T A C A G A G A C T C C A G C C C A G T 506

Germline V_h 5H 495 D S A L Y L C A V T L Y G G S G N K L I F G T G T L L S V K P 567

cDNA V_h AIO 307 G A C T C T G C T C T A T C T C T G T G C A T G A C C T T T A T G G G G C A G T G G C A A C A G C T C A T C T T T G G A A C T G G C A C T G C T T C T G T C A G C C A A 567

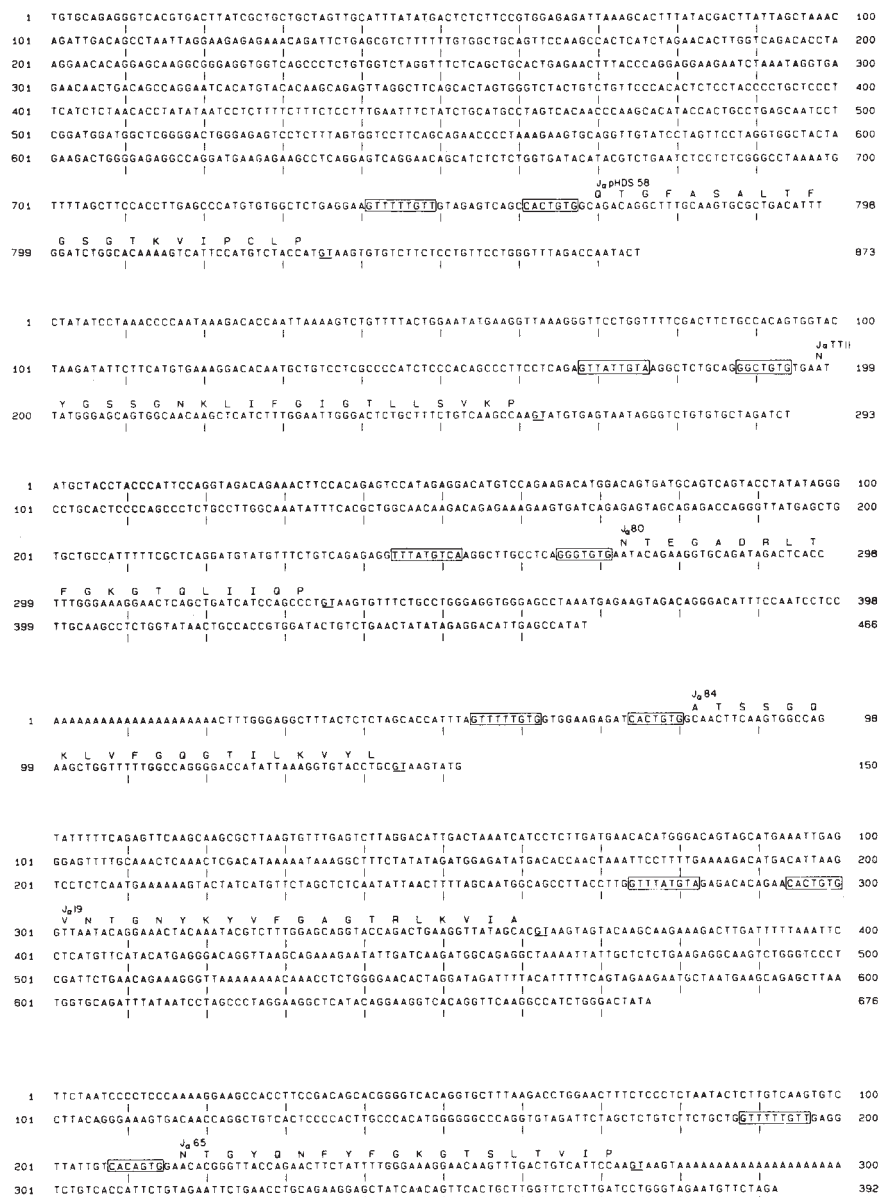
Variable Joining

TT11
Germline V_h TT11

S C S A L Y L C A V T L Y G G S G N K L I F G T G T L L S V K P
A G T G A C T C T G C T C T A T C T G T G C A T G A C C T T T A T G G G G C A G T G G C A A C A G C T C A T C T T T G G A A C T G G C A C T G C T T C T G T C A G C C A A

The presence of a D_α gene segment in a rearranged V_α gene should lead to additional nucleotide sequence between the germline V_α and J_α gene-segment sequences. The alignment of the A10 cDNA clones and the available germline V_α and J_α gene segments demonstrates that there are two extra nucleotides between the V_α and J_α gene segments in this clone (Fig. 2*b*). The J_α gene segment is the germline equivalent to that expressed

Fig. 3 DNA sequence of six germline J_α gene segments. The J gene segments are translated into protein sequence with the one-letter code. The J_α designations are given above the protein sequence. Recognition sequences for recombination are boxed and the RNA donor splicing sites are underlined. Cosmid restriction fragments containing the J_α gene segments were purified using low-melting point agarose and then subcloned into M13 bacteriophage mp18 or mp19 or both⁴⁴. Plaques of M13 containing the J_α sequences in both orientations were identified by hybridization to the J_α oligonucleotides made from DNA sequences of both strands. The following cosmid fragments were used for subcloning: 0.5-kb *Hind*III fragment for J_α TT11, 1.8-kb *Kpn*I fragment for J_α pHDS 58, 2-kb *Xba*I fragment for J_α 80, 1-kb *Bam*HI/*Hind*III fragment for J_α 84, 2-kb *Bam*HI fragment for J_α 19 and 0.5-kb *Xba*I fragment for J_α 65. DNA sequencing by the dideoxy chain termination method was done essentially as described previously⁴² and modified²⁰. Some of the DNA sequencing was done by the chemical degradation method⁴⁵. DNA sequencing was done for both strands. For every J_α -containing fragment >0.5 kb, two complementary 17-mer J_α oligonucleotides were used for the first strand sequence analysis. The opposite strands of DNA were sequenced by two additional oligonucleotides (17-mer) made according to the information obtained in the first strand DNA sequencing. Hence, at least four different oligonucleotides were made and used for sequencing one J_α gene segment to obtain information on both strands. In one case in which we could not obtain clones containing opposite orientations of the J_α gene segment (J_α TT11), the sequence was confirmed by the Maxam-Gilbert sequencing method⁴⁵.



in the A10 cDNA, whereas the germline V_α gene segment belongs to the same V_α subfamily but is not identical. In the V_κ , V_H and V_β gene families, members of the same V subfamily always end at the same codon position relative to the conserved second cysteine codon. The additional nucleotides between the rearranged V_α and J_α sequences may be explained in three ways. First, the extra nucleotides can arise from D_α gene segments that are joined to the V_α and J_α gene segments. Second, these extra sequences could arise as a result of N -region diversification. This mechanism results in the addition of random nucleotides to the 5' and/or 3' sides of the D gene segments during the joining process²³. In the immunoglobulin light-chain gene families, which do not have D gene segments, N -region diversity does not occur. Third, it is possible that the extra nucleotides are actually part of the V_α gene segment. These three possibilities cannot yet be distinguished. However, either D_α gene segments exist, N -region diversification occurs in a rearranging gene family lacking D gene segments, or the V_α gene segments within a subfamily differ in the lengths of their 3' ends.

The immunoglobulin and β -gene families use various mechanisms for somatic diversification. Junction variability occurs as a consequence of the flexibility in sites at which the gene segments are joined; and somatic hypermutation, the apparently random mutation of up to 3% of the bases in and

around a rearranged V gene, occurs in immunoglobulin genes, but rarely in β -genes^{24,25}.

The α -gene family also uses various mechanisms for somatic diversification. An alignment of the germline V_α and J_α gene segments with their cDNA counterparts revealed that there is flexibility in the sites at which the gene segments are joined together. For example, in the TA19 cDNA¹⁸, the J_α gene segment is joined five bases into the coding sequence. Thus, diversity can be introduced in the junctional region by a mechanism similar to those seen in the immunoglobulin and β -gene families. The data are inadequate to draw firm conclusions about somatic hypermutation in V_α genes because, apart from the A10 clone, these cDNA clones were derived from a thymic cDNA library. In B cells somatic hypermutation occurs late in B-cell development²⁶ and T cells in the thymus are at early stages of development. Two sets of α -gene segments do not exhibit somatic mutation. First, a comparison of the four germline J_α gene sequences analysed here with their cDNA counterparts from the same inbred strain reveals that there is no somatic mutation (see Fig. 3 and ref. 18). Second, three cDNA clones with identical V_α gene segments have been identified in the 19 different cDNA clones studied and all are found to be identical¹⁸.

The immunoglobulin and T-cell β - and γ -gene families have four distinct types of J -C organization (Fig. 4). (1) The immunoglobulin κ -genes have a cluster of four functional J

Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α -chain

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An essential property of the immune system is its ability to generate great diversity in antibody and T-cell immune responses. The genetic and molecular mechanisms responsible for the generation of antibody diversity have been investigated during the past several years^{1,2}. The gene for the variable (V) region, which determines antigen specificity, is assembled when one member of each of the dispersed clusters of V gene segments, diversity (D) elements (for heavy chains only) and joining (J) segments are fused by DNA rearrangement¹⁻³. The cloning of the β -chain of the T-cell antigen receptor^{4,5} revealed that the organization of the β -chain locus, which is similar to that of immunoglobulin genes⁶⁻¹², is also composed of noncontiguous segments of V^{7-9} , $D^{10,11}$, $J^{6,9,11}$ and constant (C) region genes⁶⁻¹². The structure of the α -chain seems to consist of a V and a C domain connected by a J segment¹³⁻¹⁶. We report here that the human T-cell receptor α -chain gene consists of a number of noncontiguous V and J gene segments and a C region gene. The V region gene segment is interrupted by a single intron, whereas the C region contains four exons. The J segments, situated 5' of the C region gene, are dispersed over a distance of at least 35 kilobases (kb). Signal sequences, which are presumably involved in DNA recombination, are found next to the V and J gene segments.

Southern gel analysis¹⁷ was performed using human genomic DNA and nucleotide probes corresponding to the V, J and C region sequences of a human T-cell receptor α -chain cDNA, pY14 (ref. 16). We find that the entire pY14 insert hybridizes to ~10 fragments when genomic DNA is digested with *EcoRI*, *BamHI*, *HindIII* or *BglIII* (Fig. 1). Using a C-region-specific probe or J-region-specific probe, only one or two of these bands can be assigned to the C and J sequences (data not shown), whereas the rest correspond to fragments homologous with V gene segments. These results suggest the presence of only one C region and multiple V gene segments.

Seventeen recombinant phage from a human genomic library (from ref. 18) carrying inserts that hybridized to the V region of pY14 (*V_αpY14.1*) under non-stringent hybridization conditions were isolated. The nucleotide sequence was determined for one clone (λV13). Figure 2 shows the nucleotide sequence of this V gene segment, *V_αpY14.2*, which is divided into two exons. The first exon stretches from amino acids 1 to 15 and includes most of the presumptive signal peptide, whereas the second exon begins at amino acid 16 and ends at amino acid 113. The intron is 101 nucleotides long. Sequences that resemble the heptamer (CACAGTG) and nonamer (ACACAAACC) recognition signals that are involved in the recombination of immunoglobulin¹⁻³ genes and T-cell receptor β-chain genes⁶⁻¹² are next to the 3' end of the second exon, and are separated by 22 nucleotides. The complementary DNA sequence *V_αpY14.1* and the germline sequence *V_αpY14.2* are identical at 251 out of 279 nucleotides (90%) in the protein-coding region, and are identical at 76 out of 93 amino acids (82%). This may not be the gene segment that encodes the pY14 V region.

The results of Southern blotting (see Fig. 1) suggest that there is only one germline C_α gene. To characterize the C region at the molecular level, four λ clones that hybridize with the C region were isolated. Two of these (clones 8 and 18) also hybrid-

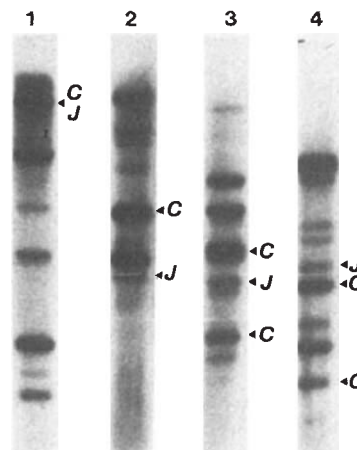


Fig. 1 Southern gel analysis of the *V*, *J* and *C* region genes in human genomic DNA. DNA was digested with restriction enzymes, *Eco*RI (lane 1), *Bam*HI (lane 2), *Hind*III (lane 3) and *Bgl*II (lane 4) and analysed by Southern blot^{6,17} using radioactive probes from: (1) the complete pY14 cDNA from Jurkat T-cell line (ref. 16); (2) an oligonucleotide corresponding to the *J* region sequence (nucleotides 545–593 in Fig. 4 of ref. 16); and (3) the *C* region probe alone (*Hpa*II/*Hpa*II fragment at nucleotides 591–980 in Fig. 4 of ref. 16). The results using the complete pY14 cDNA probe are shown, but the fragments corresponding to the *C* and *J* segments are denoted.

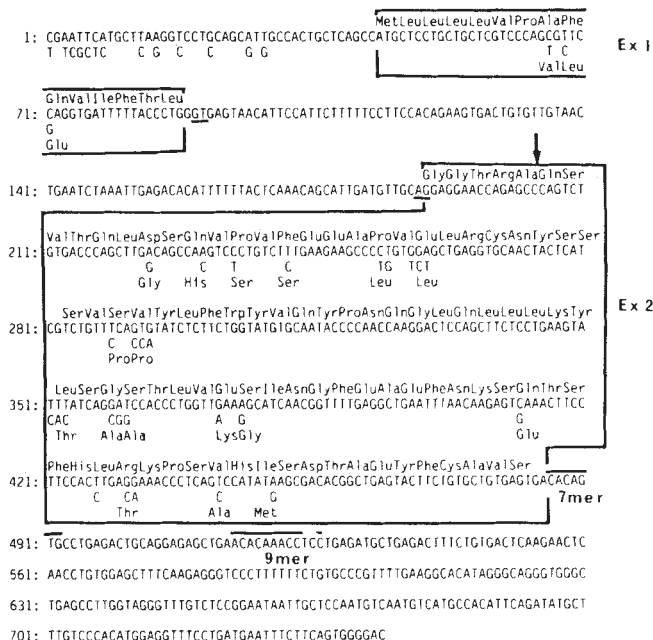


Fig. 2 Nucleotide sequence of a germline V segment $V_{\alpha}pY14.2$ of the human T-cell receptor α -chain genes. Seventeen λ recombinant clones in the human genomic library¹⁸ homologous with the V sequences of pY14 (ref. 16), *Eco*RI/*Sau*3A fragment of pY14 (nucleotides 1–529 in Fig. 4 of ref. 16) were identified by hybridization and washed at 5 $\times$ SSC, 42 $^{\circ}$ C. One clone, λ V13, which contains a 1.3-kb *Eco*RI fragment, hybridized in stringent conditions (0.11 $\times$ SSC, 68 $^{\circ}$ C). The insert was sequenced using the Sanger dideoxy method²⁴. For the detailed sequencing procedure, see ref. 25. Ex, exon with recombinational signals overlined; underline, splicing signals of AG and GT; arrow, end-of-signal peptide. Differences in nucleotide and deduced amino acids of $V_{\alpha}pY14.1$ from cDNA pY14 (ref. 16) are denoted below the sequences of $V_{\alpha}pY14.2$.

ized to a *J* sequence probe of pY14 (*J_αpY14*). Using the technique of 'chromosome walking', λ -clones 21 and 22 were also obtained. A detailed restriction enzyme map of these clones was determined (Fig. 3A). Regions that hybridized with oligonucleotides to α -chain human and murine *J_α* sequences (refs 13-16) are also indicated. The *C* region is mainly contained on a 5.7-kb

[illegible]

The regions of λ -clones 8, 21 and 22 that hybridize to J_α probes are indicated in Fig. 3A. The 3'-most J region that was identified lies close to the 5' end of a 3.6-kb *Bam*HI fragment ~4 kb upstream of the C_α gene. This J region hybridizes to a J_α pY14 probe under stringent conditions. The sequence of the 3.6-kb *Bam*HI fragment is presented in Fig. 5a. A sequence identical to the J segment of pY14 is found at nucleotides 509–565. Immediately 5' are the heptamer and nonamer recombination signals (CACAGTG and GGTTATCTC, respectively) separated by a 12-nucleotide-long spacer. These sequences are the same as the signal sequences for recombination that are found in immunoglobulin¹⁻³ and the β -chain of the T-cell receptor⁶⁻¹². Examination of the 3.6-kb sequence indicates that two other sequences can be found, including the highly conserved central Phy-Gly-X-Gly sequence. The first of these, from 1,407 to 1,462, is unlikely to be a functional J element because the putative splice donor site is not positioned to maintain the proper reading frame when joined to the C_α region. It also has poorly recognizable heptamer and nonamer signal sequences. The second other potential J_α segment, from 2,367 to 2,423, matches J_α pY14 at 9 of 19 amino acids and has a potential splice donor site at the proper position; however, the heptamer and nonamer sequences do not match the expected sequence very well. An *Alu* repetitive element spans positions 970–1,200 of the 3.6-kb *Bam*HI fragment. Hybridization analysis using oligonucleotide probes to murine and human J sequences indicates that ~12 human J_α sequences (Fig. 3A) may be scattered over these three

[illegible]

λ clones (8, 21 and 22). To confirm that at least some of these are J sequences, regions denoted b , c , d , e and f in Figure 3A were also sequenced. Their J sequences are illustrated in Fig. 5b–f. It is clear that all these sequences have easily identifiable heptamer and nonamer recombination signal sequences; the highly conserved central Phy-Gly-X-Gly and splice sequences at the 3' end match the concessive sequence well. The sequence illustrated in Fig. 5f is not complete as it is at the end of clone 22. More recent experiments also show that J_{α} sequences extend further 5' to these clones. These J_{α} segments are longer than

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Smooth muscle α -actin is a transformation-sensitive marker for mouse NIH 3T3 and Rat-2 cells

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Heteroploid mouse NIH 3T3 fibroblasts and several rat fibroblast strains (Rat-1, Rat-2 and REF-52) are cell lines of special interest in the field of carcinogenesis because of their extensive use as normal cells in transformation assays for putative cancer-causing genes. Exposure of these cells to carcinogenic chemicals or oncogenic DNA produces anchorage-independent cells with retracted cytoplasm that lack actin cables¹⁻⁷. All human fibroblast strains, normal and transformed, synthesize two electrophoretic forms of actin (β - and γ -actin)⁸⁻¹⁰. In contrast, we discovered that early-passage mouse and rat strains synthesize abundant amounts of each of the three electrophoretic forms of actin (α -, β - and γ -actin) but mouse and rat cancer cells express only β - and γ -actins. We now show that in NIH 3T3 and Rat-2 fibroblasts a third actin, the smooth muscle α isoform, is abundantly co-expressed with β - and γ -actin. In every instance tested following transformation to tumorigenicity, the accumulation of α -actin messenger RNA and α -actin synthesis was greatly inhibited. Shut-down of α -actin expression thus appears to be a reproducible transformation-sensitive marker in rodent fibroblasts.

Figure 1 shows the actin peptides made in untransformed and transformed NIH 3T3 and Rat-2 cells. Using computerized microdensitometry¹¹, we estimate that NIH 3T3 cells synthesize a considerable amount of α -actin, about 25-30% as much as β -actin, and Rat-2 cells synthesize about 25-40% as much α -actin as β -actin. All 68 Rat-2 subclones examined synthesized more α -actin than the parental Rat-2 strain (Fig. 1). The ratio of the rates of α -actin to β -actin synthesis increased from 0.25 to 0.42 as a consequence of subcloning. We and others⁵ have observed that Rat-2 subclones exhibit flatter, more contact-inhibited monolayers than the parental Rat-2 strain. Thus elevation in the rate of α -actin synthesis is associated with the acquisition of the flatter, more contact-inhibited phenotype on subcloning of Rat-2 cells.

We isolated transformed NIH 3T3 or Rat-2 cell lines from rare foci that arose spontaneously in confluent monolayers. Two such spontaneously transformed cell lines, T-3T3 and FocT, exhibit no α -actin (Fig. 1). We excluded the possibility that these spontaneous transformants arose from a subpopulation of non-transformed cells that do not make α -actin by screening 68 subclonal Rat-2 lines. Each line synthesized α -actin at about the same rate as the Rat-2 subclone shown in Fig. 1. Thus α -actin is a uniformly abundant gene product in Rat-2 cells and in NIH 3T3 cells.

A similar loss of α -actin was detected in all *in vitro*-transformed cell lines that we have examined to date. These additional lines include four separate EJ-Ha-ras oncogene transformed⁴ NIH 3T3 lines, one additional spontaneous transformant of Rat-2, two separate Rat-2 strains transformed with EJ-Ha-ras oncogene, a line transformed by transfection of Rat-2 cells with

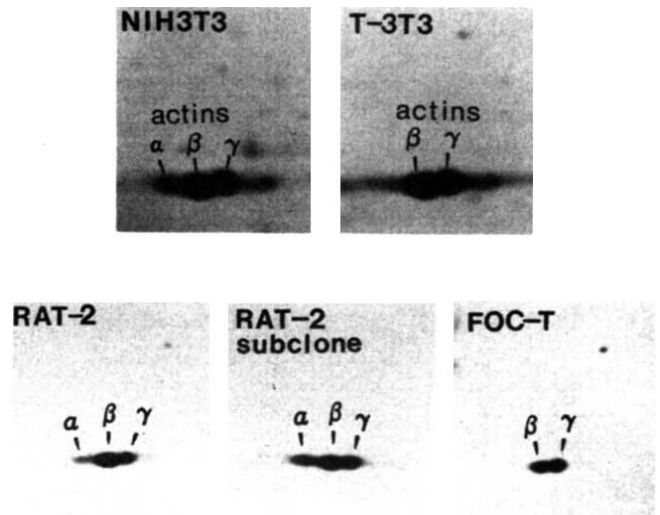


Fig. 1 Autoradiograms of the cellular α -, β - and γ -actin polypeptides of normal and transformed NIH 3T3 and Rat-2 cells separated and resolved by two-dimensional polyacrylamide gel electrophoresis. An ampoule of frozen NIH 3T3 cells² was obtained from Dr Brian Crawford (Los Alamos National Laboratory) who received his 3T3 stock from Dr Esther Chang (Uniformed Services Medical School, Bethesda). The 3T3 stock was cultured through two passages at Los Alamos before preparation of the frozen ampoule used as the seed stock for the present study. The Rat-2 cell line¹ was from Dr Greg Reyes (Stanford University) who obtained his stock from Dr Mike Botchan (University of California, Berkeley). T-3T3 and FocT cell lines were derived from spontaneous foci that appeared in confluent monolayers of NIH 3T3 and Rat-2 cells, respectively. The two focus-derived cell lines were isolated from the focus by adsorption to a 3-mm Whatman filter paper disk which had been soaked in EDTA-trypsin solution (0.25% trypsin, 0.1 mM EDTA). Both cell lines are composed of highly refractile, morphologically transformed cells that produce rapidly growing tumours visible 1 week after subcutaneous inoculation of 2×10^6 cells into nude mice or syngeneic animals. The 'Rat-2 subclone' was a hypoxanthine-aminopterin-thymidine (HAT)-resistant clonal cell line induced by transfection of Rat-2 cells with a cloned thymidine kinase gene (J.L. and R.J., in preparation). All cell cultures were labelled with ³⁵S-methionine for 4 h⁹. Total proteins were solubilized in Lysis A solution^{9,10,24} and the actin polypeptides separated by two-dimensional electrophoresis²⁴. Identification of the actin polypeptides is based on their abundance and relative position in the two-dimensional gel.^{8-10,14,17,18,24,25}

a cloned transforming DNA fragment (*Bam*HI-E) from human herpes simplex virus type 2 (ref. 12 and B. Tanczos *et al.*, in preparation), and four separate transformed cell lines induced by transfection of Rat-2 cells with a combination of cloned transforming DNA fragments (*Xba*I/*Bam*HI fragments EJ and EM) from human cytomegalovirus strain Towne (ref. 13 and R.J. *et al.*, in preparation).

In addition, Franza and Garrels¹⁴ have reported the same finding for rat embryo fibroblasts (REF-52 strain) transfected with the transforming genes of human adenovirus, Kirsten murine sarcoma virus, and simian virus 40. Mouse L-cells, which are highly transformed fibroblasts, also lack α -actin¹⁵.

To confirm α -actin expression in NIH 3T3 and Rat-2 cells, we demonstrated that an α -like actin RNA transcript is present in these cells but is greatly diminished or absent from the transformed cell lines. Figure 2 shows RNA transfer blot hybridization experiments in which total RNA from each cell type has been separated electrophoretically and then hybridized with isotype-specific RNA probes¹⁶ for β -actin (Fig. 2b) or γ -actin (Fig. 2c), or an actin coding sequence probe (Fig. 2a, d). mRNA transcripts for β - or γ -actin are 2,100 base pairs (bp) long^{15,16}. RNA transcripts that code for the three muscle-related α -actins are about 1,600-1,700 bp long¹⁵⁻¹⁸. With the β - and γ -actin-specific probes, only a 2,100-bp transcript was found

Table 1 Modulation of actin mRNA synthesis

		Relative amounts of α -, β - and γ -actin mRNAs			
		Coding probe*	β - and γ -actin isotype-specific†		γ -mRNA β -mRNA modulation‡
		α -mRNA	β -mRNA	γ -mRNA	
		β - plus γ -mRNA			
Mouse (expt 1):					
NIH 3T3	Normal	0.390	1.00	1.00	1.00
T-3T3	Spontaneously transformed§	0.040	2.12	1.16	0.55
Rat (expt 1):					
Rat-2	Normal	0.110	1.00	1.00	1.00
Rat-2 subclone	Normal HAT ^r , HSV-2 tk gene transfectant	0.080	0.89	0.88	0.99
FocT	Spontaneously transformed§	<0.002	0.86	11.47	13.34
Rat (expt 2):					
Rat-2	Normal	0.094	1.00	1.00	1.00
FocT	Spontaneously transformed§	<0.002	0.68	5.65	8.31
RBET	HSV-2-transformed	<0.002	0.73	2.19	3.00
RXBJMT1	CMV-transformed¶	<0.002	0.84	2.07	2.46
RXBJMT2	CMV-transformed	<0.002	0.74	1.79	2.41
RXBJMT3	CMV-transformed	<0.002	0.69	1.04	1.51
RXBJMT4	CMV-transformed	<0.002	0.93	1.64	1.76

Relative amounts of the actin mRNAs were measured by hybridization with actin-coding or isotype-specific ³²P-DNA probes.

* Relative hybridization of an actin-coding region ³²P-DNA probe (Fig. 2) to either the 2,100-bp β -plus γ -actin mRNA band or the band representing the 1,600–1,700-bp α -actin in the RNA transfer blots shown in Fig. 2a,d; unsaturated autoradiographic bands resulting from α -specific or β -, γ -specific hybridization were quantified by computerized microdensitometry and the ratio of these measurements is shown.

† Hybridization of β -actin-specific ³²P-DNA probe to the 2,100-bp β -actin mRNA shown in Fig. 2b was quantified by computerized microdensitometry; the amount of hybridization for each total RNA isolate is expressed as a ratio—the amount of hybridization to mRNA from the derivative cell line relative to the amount of hybridization to mRNA from the parental cell line, either NIH 3T3 or Rat-2; or hybridization of the γ -actin-specific ³²P-DNA probe to 2,100-bp γ -actin mRNA as shown in Fig. 2c.

‡ Ratio of γ -actin-specific hybridization (Fig. 2c) to β -actin-specific hybridization (Fig. 2b), or the modulation in amount of γ -actin mRNA accompanying transformation if the amount of β -actin mRNA is kept constant.

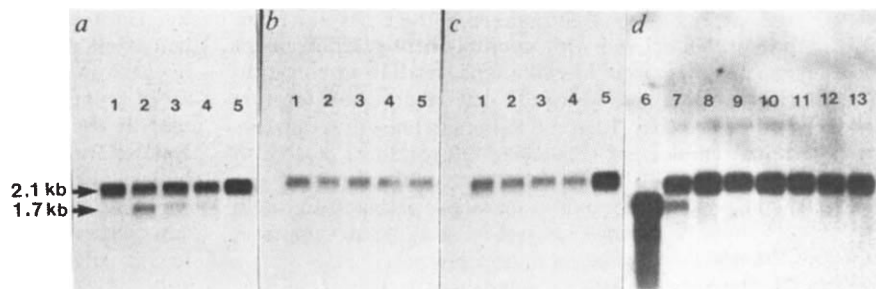
§ Derived from a focus that arose spontaneously in a confluent Rat-2 or NIH 3T3 cell monolayer.

|| *Bam*HI fragment E (ref. 12).

¶ Mixture of *Xba*I/*Bam*HI fragments EJ and EM¹³.

Fig. 2 Autoradiograms of total RNA transfer blots hybridized with ³²P-DNA probes derived from an actin-coding sequence or isotype-specific 3'-untranslated region (3'UTR) sequences. In a and d, the probe was a *Pvu*II/*Xba*I DNA fragment from pHM α A-1 (ref. 27) containing the coding region of human skeletal α -actin from amino acid 202 to amino acid 374. This probe hybridizes equally well with muscle and non-muscle actin mRNAs (data not shown). In b, the probe was the human β -actin-specific subclone pHF β A-3'UT¹⁶ and in c the probe was the human γ -actin-specific subclone pHF γ A-3'UT¹⁶. The cell source of total RNA in each electrophoretic lane is as follows: lane 1, T-3T3; lane 2, normal NIH 3T3; lanes 3 and 7, normal Rat-2; lane 4, Rat-2 subclone (Fig. 1); lanes 5 and 8, FocT; lane 6, mouse skeletal muscle^{15,16}; lane 9, RXBJMT1 (Table 1); lane 10, RBET (Table 1); lane 11, RXBJMT2 (Table 1); lane 12, RXBJMT3 (Table 1); and lane 13, RXBJMT4 (Table 1).

Methods. Total RNA (2 μ g) was isolated from each cell line, size-fractionated on 2.2 M formaldehyde–1% agarose gels and blot-transferred to nitrocellulose filters as described previously²⁸. The filters were hybridized with one of three different nick-translated²⁹ DNA probes. Hybridization was done in 4 $\times$ SSC, 50 mM Na₂HPO₄ (pH 7.0), 5 $\times$ Denhardt's³⁰, 10% (w/v) dextran sulphate containing 10⁶ d.p.m. radiolabelled DNA probe per ml at 65 °C. Following hybridization, the filters were washed with five changes of 0.5 $\times$ SSC, 0.1% (w/v) SDS and exposed to Kodak XAR-5 film.



(Fig. 2b,c). With the nonspecific actin-coding probe, an abundant, 1,600–1,700-bp transcript is detected in normal NIH 3T3 and Rat-2 cells, together with the 2,100-bp transcripts for β - and γ -actin (Fig. 2a,d). This result firmly establishes the presence of a third expressed actin mRNA in these cells. The presence of a third actin mRNA also excludes the possibility that the α -actin identified in two-dimensional gels was a post-translationally modified product of β - or γ -actin. Perhaps a trace of α -like transcript is detectable in the transformed NIH 3T3 strain (Fig. 2a, lane 1) but no α -like transcript is found in the transformed Rat-2 strain (Fig. 2a, lane 5). In addition, the herpes simplex virus 2- and four cytomegalovirus-trans-

formed Rat-2 cell lines contain no α -like transcript (Fig. 2d, lanes 9–13; Table 1).

In an attempt to identify which α -actin isoform is expressed in NIH 3T3 and Rat-2 cells, we hybridized replicate RNA blots with 3'-untranslated region probes specific for α (skeletal)- and α (cardiac)-actin mRNA¹⁶; no transcripts were detected (not shown). Thus we conclude by elimination that the α -actin expressed in NIH 3T3 and Rat-2 cells is not β -, γ -, α -skeletal or α -cardiac and is probably the α -actin made in smooth muscle¹⁹. We cannot exclude the formal possibility that the α -actin is an as yet undefined α -actin isotype. However, Vanderkerckhove and Weber²⁰ have demonstrated that cultured

chick embryo fibroblasts synthesize smooth muscle α -actin in addition to non-muscle β - and γ -actin. This prior observation strongly supports the conclusion that the α -actin we detect in rodent fibroblast cell lines is also of the smooth muscle type.

In the spontaneously transformed Rat-2 cell line FocT there was an additional unexpected change in actin expression. Rat-2 cells accumulate less γ -actin than β -actin (Fig. 1), so that the ratio of γ -actin to β -actin is 0.4. The spontaneously transformed Rat-2 cell line accumulates more γ -actin than β -actin, amounting to a threefold up-regulation of γ -actin relative to β -actin (Fig. 1). The amount of γ -actin mRNA is up-regulated even more dramatically relative to the β -actin mRNA (Fig. 2b, c, lane 5). Assuming that these changes are not related to altered mRNA stability, we estimate that the rate of γ -actin gene transcription has increased 8–13-fold in this transformed strain (Table 1).

To examine the relative significance of the actin protein modulations, we have analysed the 500 most abundant polypeptide species in high-resolution two-dimensional gels of total proteins from most of the normal and transformed Rat-2 cells discussed in the present paper. We consistently find no qualitative changes in polypeptide synthesis by Rat-2 cells other than the loss of α -actin. The same finding is implied by Franza and Garrels¹⁴, although they and Matsumura *et al.*²¹ also report that the microfilament-associated tropomyosin subunits of REF-52 are modulated quantitatively but variably among different types of transformed cells. Thus, at least among the 500 abundant cellular proteins of rat fibroblasts, the alterations of actin expression are not a general phenomenon shared by other proteins.

Rat-2 and NIH 3T3 cells are similar in their shape and relatively flat morphology¹, they are both aneuploid^{1,2}, and will consistently form subcutaneous tumours in syngeneic animals, but only after long latent periods of 10–12 weeks (J.L. and R.J., unpublished results). By contrast, all of the transformed cell lines mentioned in this study produce, within 1–3 weeks, solid tumours that grow rapidly to a mass 4 cm in diameter by 4–5 weeks. Morphologically these transformed cells are typical of other transformed cells^{3–8,12–14,22} but contrast with untransformed Rat-2 and NIH 3T3 cells in that their cytoplasm is retracted and refractile on the plastic substratum. Thus modulations in actin expression and abundance of the types described in this study are associated with microfilament rearrangement and subsequent alterations in cell shape, leading to elevation of tumorigenic potential. Indeed, one acute transforming oncogene of the Gardner-Rasheed feline sarcoma virus encodes the N-terminal amino-acid sequence (130 residues) of actin²³. This finding supports the hypothesis that alteration of the microfilament system through qualitative changes in actin expression^{8–10,14,22–26} or organization^{7,8,26} may be an important aspect of the neoplastic transformation process.

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Detection of deletions spanning the Duchenne muscular dystrophy locus using a tightly linked DNA segment

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The Duchenne muscular dystrophy (DMD) locus has been localized to the short arm of the human X chromosome (Xp21) by detection of structural abnormalities^{1–5} and by genetic linkage studies^{6–8}. A library highly enriched for human DNA from Xp21 was constructed⁹ using DNA isolated from a male patient who had a visible deletion and three X-linked disorders (DMD, retinitis pigmentosa and chronic granulomatous disease)⁵. Seven cloned DNA probes from this library and the probe 754 (refs 5, 8) are used in the present study to screen for deletions in the DNA isolated from 57 unrelated males with DMD. Five of these DMD males are shown to exhibit deletions for one of the cloned DNA segments and at least 38 kb of surrounding DNA. In addition, two subclones from the same region detect four restriction fragment length polymorphisms which exhibit no obligate recombination with DMD in 34 meiotic events. These new DNA segments will complement the existing Xp21 probes^{4–9} for use in carrier detection and prenatal diagnosis of DMD. Elucidation of the end points of the five deletions will help delineate the extent of the DMD locus and ultimately lead to an understanding of the specific sequences involved in DMD.

The DNA of 57 unrelated DMD males was tested for deletions of the following 8 cloned DNA segments absent from the male patient with a visible deletion⁵: pERT55-2, pERT84, pERT87, pERT145, pERT378, pERT379, pERT469 and 754 (pERT, plasmids isolated from a library enriched for Xp21 DNA segments⁹). Based on a previously described strategy, 8 probes from a small region of the genome should increase the probability that one probe will detect a small deletion at a specific locus in the region¹⁰. Among the 8 clones tested, only one, pERT87, was found to be absent from the DNA of 5 of the 57 unrelated DMD males.

pERT87 was screened by hybridization¹¹ against total human genomic libraries constructed in Charon 35 (ref. 12) and EMBL-3 (ref. 13) to obtain more surrounding DNA. After chromosomal

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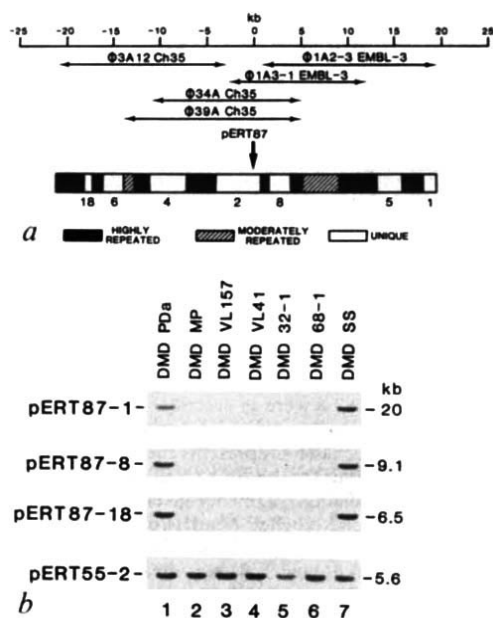


Fig. 1 **a**, Schematic map of 41 kilobases of genomic DNA around the original 200 base pairs, pERT87 (0 on kb scale). The subcloned segments that were free of human repeated sequence elements are indicated numerically below the block diagram, and the size of the segment is represented by an open block. Segments containing highly repeated sequences are indicated as darkened areas, and those containing moderately repeated segments as cross-hatched areas. **b**, Southern blot¹⁴ analysis of DNA from seven males with DMD. Lanes 1-7 contain *Pst*I-digested DNA of the patients. Two identical nitrocellulose filters were hybridized with pERT87-18 and pERT87-1. The same filters were then rehybridized with pERT87-8 and pERT55-2, respectively. The autoradiographs are presented as separate segments of each original autoradiograph. The fragment hybridized is indicated to the left of the figure; the sizes of hybridizing fragments are indicated on the right and were calculated from ³²P-end-labelled *Hind*III-cleaved λ DNA. The numerical designation of the subclone used as a probe and the probe's relative position in 'walked' DNA are indicated in **a**. The designation above each lane is the nomenclature for each DMD male.

Methods. Five overlapping bacteriophage clones (lines with arrows below kb scale) were isolated¹¹ from two total human DNA recombinant libraries constructed in Charon 35 (ref. 12) and EMBL-3 (ref. 13) (generously provided by Drs S. Orkin and Y. Shiloh, respectively). Each library was constructed from size-selected (13–21 kb) partially *Mbo*I-digested human DNA and the respective phage vector. Screening¹¹ was against nitrocellulose filters lifted from primary plates (the libraries were never amplified). Hybridizing phage were plaque-purified and *Hind*III-digested human DNA segments were subcloned²² into *Hind*III-cleaved and dephosphorylated pBR322 (New England Biolabs). The subclones were digested with various restriction enzymes (*Eco*RI, *Xba*I and *Kpn*I) to yield smaller fragments free of human repetitive sequence elements. Genomic DNA was isolated from cell nuclei of whole blood leukocytes as described previously⁷ and digested with *Pst*I in buffers recommended by the manufacturer. The digested DNA (1.5 μ g) was separated by electrophoresis on a horizontal 0.7% agarose gel and transferred to nitrocellulose¹⁴. The unique-sequence subclones pERT87-18, pERT87-8, pERT87-1 and pERT55-2 were ³²P-radiolabelled using T4 DNA polymerase and radiolabelled fragments were recovered from low melting point agarose gels⁷. Prehybridization of nitrocellulose filters was undertaken as described previously⁷ and hybridization was at 45 °C in 50% deionized formamide, 4 $\times$ SSC (1 $\times$ SSC=0.15 M NaCl, 0.015 M Na citrate), 0.05 M sodium phosphate buffer (pH 6.8), 8% dextran sulphate, 10 $\times$ Denhardt's, 100 μ g ml⁻¹ transfer RNA, 25 μ g ml⁻¹ sheared salmon sperm DNA, 0.1% SDS, 1 mM EDTA and 10⁶ c.p.m. ml⁻¹ of radiolabelled DNA. Filters were washed in 0.1% SDS and 0.1 $\times$ SSC several times at room temperature and then for 1 h at 55 °C. Autoradiography was at -70 °C with a DuPont intensifying screen for 1–5 days.

'walks' in both directions from the original pERT87 clone, five bacteriophage clones were isolated which had a combined insert size of 41 kilobases (kb) (Fig. 1a). Small unique-sequence subclones from the recovered pERT87 human inserts were hybridized¹⁴ to *Pst*I- or *Taq*I-cleaved DNA of the 57 DMD males. The results for the five deletions are presented in Fig. 1b together with results from two DMD boys who do not bear deletions of the pERT87 region. The subclones pERT87-18, pERT87-8 and pERT87-1 are absent from the DNA of these five unrelated individuals (Fig. 1b, lanes 2–6). These three subclones span a region of 38 kb of genomic sequences (see schematic map, Fig.

Table 1 Detection of deletions in DMD males

	Total	Deletions
DMD X chromosomes		
Familial origin	17	3 (18%)
Sporadic origin	40	2 (5%)
Total	57	5 (9%)
Normal X chromosomes	61	0

The DNA isolated from 57 DMD males, 47 normal males and 7 normal females was cleaved with *Pst*I or *Taq*I. Diagnosis of DMD was established by characteristic history, physical examination, increased CPK levels and a positive muscle biopsy²¹. None of the five boys with deletions exhibited mental retardation, developmental delay, recurrent infections or retinal abnormalities. Following separation of digested DNA by electrophoresis, the DNA samples were transferred to nitrocellulose and hybridized with pERT87 phage subclones or other Xp21 cloned DNA fragments as described in Fig. 1 legend. All 57 DMD X chromosomes were tested for deletion of the pERT87 region, as were all 61 normal X chromosomes. In some cases only 37 DMD males were tested for deletion of other pERT clones or 754, but all 5 boys deleted for the pERT87 region were tested for deletion at other pERT and 754 loci. Only the pERT87 region was found to be deleted in any of the 57 DMD males. Deletions were defined as complete absence of hybridization of a cloned fragment to any restriction fragment in a particular endonuclease digest. In all cases at least one other pERT clone or 754 was shown to be present on the same nitrocellulose filter on which the pERT87 region exhibited deletion. Familial cases of DMD were determined either by extensive family history of DMD or, in a few cases, by elevated CPK^{15,16} in mothers of DMD males. Sporadic cases were those where no elevated CPK was observed in a mother with no family history of the disease. Normal individuals were defined as fathers of males with DMD or other X-linked diseases, random normal individuals or, in some cases, individuals affected by other X-linked diseases.

1a) and therefore give a lower limit of 38 kb to all five deletions. If the deletion in the male patient⁵ lacking all eight DNA fragments is 2,000–5,000 kb and if the eight cloned fragments are uniformly distributed within the deleted region in normal DNA, then an upper estimate of the five deletions detected by pERT87 is 250–600 kb.

The five deletions detected represent 9% of DMD males tested. Of 61 normal X chromosomes (47 males and 7 females), no deletions were found in the pERT87 region (Table 1). Among the boys who were found to have deletions, two were sporadic cases of DMD with no previous family history. The other three were familial cases of DMD (determined by elevated creatine phosphokinase (CPK) levels in suspected carrier females^{15,16} or a family history of DMD). The results given in Table 1 and Fig. 1b are consistent with results obtained with two other X-linked disorders, Lesch-Nyhan syndrome¹⁷ and ornithine transcarbamylase (OTC) deficiency¹⁸, in which 18 and 6.6% of patients were found to have partial or complete deletions of genomic DNA sequences complementary to the cloned cDNAs for hypoxanthine-guanine phosphoribosyltransferase and OTC, respectively.

Several unique subclones from the pERT87 region were hybridized to nitrocellulose filters containing immobilized DNA samples isolated from four unrelated females cleaved with 24 different restriction enzymes⁷. The subcloned probe pERT87-8 (a 1.3-kb *Xba*I fragment next to the original pERT probe, pERT87; see Fig. 1a) was found to recognize both a *Bst*XI and a *Taq*I restriction fragment length polymorphism (RFLP). Figure 2a shows the segregation of the *Bst*XI RFLP and demonstrates mendelian inheritance with no obligate recombinants in five meiotic events. The segregation of the *Taq*I RFLP (Fig. 2) shows no obligate recombination in six meiotic events. To date, the 2 RFLPs seen by pERT87-8 have not recombined in 34 meiotic events in 10 DMD families. A lod score was calculated¹⁹ based on segregation results of the two pERT87-8 RFLPs in these 10 DMD families (two presented here); there were seven meioses in which the phase was known and 24 in which it was not known. The only female individuals who were judged to be carriers or normal on the basis of CPK levels are those presented

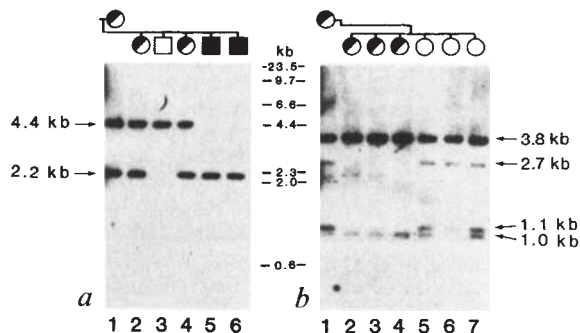


Fig. 2 Segregation of pERT87-8 in DMD families. **a**, DNA was isolated from individuals within a family in which the DMD trait segregated (indicated by darkened symbols), and the DNA was cleaved with *Bst*XI. Electrophoresis, blotting and hybridization were as described for Fig. 1b. On *Bst*XI digestion of genomic DNA, pERT87-8 hybridizes to 4.4- and 2.2-kb *Bst*XI fragments in females heterozygous for the RFLP. The 4.4-kb fragment is observed in 60% of X chromosomes and the 2.2-kb fragment in 40% of X chromosomes (28X chromosomes tested). The carrier mother exhibits the heterozygous pattern of the 4.4/2.2-kb *Bst*XI alleles, as do her two carrier daughters (lanes 2, 4). The lower allele (2.2 kb) is present in both affected DMD probands (lanes 5, 6) while the upper allele (4.4 kb) is present in the unaffected son (lane 3). The father in this family has a 4.4-kb *Bst*XI hybridizing fragment (data not shown). The female individuals in lanes 2 and 4 were suspected of being carriers of the DMD trait based on elevated CPK values^{15,16,21}. Fragment sizes were calculated by comparison with ³²P-end-labelled *Hind*III-cleaved λ phage DNA (shown in centre of figure). **b**, The DNA isolated from individuals of a family segregating the DMD trait was cleaved with *Taq*I. The pERT87-8 probe hybridizes to a 3.8-kb, 2.7-kb, 1.1-kb and 1.0-kb fragment in females heterozygous for the RFLP. The 1.0-kb *Taq*I fragment is present in all individuals, whereas the RFLP is revealed by the presence or absence of a *Taq*I site on a 3.8-kb fragment. The rarer allele is a 3.8-kb fragment (27% of X chromosomes), and the common allele is represented by 2.7- and 1.1-kb fragments (73% of X chromosomes tested). The carrier mother (lane 1) exhibits the heterozygous *Taq*I pattern of the 1.1-, 2.7/3.8-kb bands. Her three DMD carrier daughters (lanes 2-4), all of whom have affected sons, show two-copy hybridization to the large 3.8-kb allele, while the three suspected non-carrier daughters (based on their CPK values; lanes 5-7) exhibit the heterozygous pattern. This demonstrates that the X chromosome with the 3.8-kb allele from the carrier mother is inherited by the three carrier daughters along with the 3.8-kb allele from the father. The affected sons of these carrier women all inherited a 3.8-kb and a 1.0-kb *Taq*I fragment from their mothers (data not shown). Females were determined to be carriers of the DMD trait by elevated CPK and sons with DMD; non-carrier women were determined by normal CPK. The dark smudges between 2.7 and 1.1 kb in lanes 1-5 are nonspecific and are caused by the need to autoradiograph the nitrocellulose filter for 6 days to visualize the faintly hybridizing 2.7-, 1.1- and 1.0-kb fragments. These only occurred in this autoradiograph.

in Fig. 2; most results were obtained with affected or normal sons of obligate heterozygous DMD females. The coincidence of deletions for pERT87 and the DMD trait was not used in the calculation of lod scores. The calculated maximum lod score was 7.6 at $\theta = 0$ centimorgans at confidence limits, set by lod scores one unit lower, of 0 to 7 centimorgans. A more precise recombination fraction will take many more informative meioses.

To characterize more precisely the sporadic or familial nature of the deletions detected by the pERT87 probes, the *Bst*XI RFLP was used to tag specific X chromosomes in two families containing DMD boys bearing deletions. In the first family (sporadic), the mother (Fig. 3a, lane 5) of a DMD boy with a deletion (Fig. 3a, lane 4) is heterozygous for the *Bst*XI RFLP. This woman must have two copies of the pERT87-8 locus and is not heterozygous for the deletion. In the second family (familial), the grandmother (Fig. 3b, lane 1) is either hemizygous or homozygous for the 2.2-kb *Bst*XI allele, but her daughter (Fig. 3b, lane 2) has only the 4.4-kb allele, presumably inherited from her father. As the daughter has no hybridizing 2.2-kb allele which she should have inherited from her mother and as the hybridization intensity of the 4.4-kb allele is similar to that of a single X chromosome in the control female (Fig. 3b, lane 5), we presume that the daughter is heterozygous for the deletion evident in her two DMD sons (Fig. 3b, lanes 3, 4).

The *Bst*XI and *Taq*I RFLPs are predicted to be heterozygous in 48 and 39%, respectively, of women tested (allele frequencies

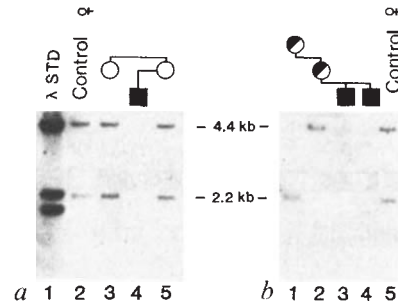


Fig. 3 Analysis of deletions for pERT87-8 in DMD families. **a**, DNA was isolated from a mother of a DMD boy with a deletion and from her sister. Electrophoresis of *Bst*XI-cleaved DNA and hybridization of pERT87-8 were as described in Fig. 1 legend. The open circles above lanes 3 and 5 indicate the two sisters and the darkened symbol above lane 4 represents the DMD son. An unrelated female heterozygous for the *Bst*XI RFLP is presented in lane 2. The sizes of hybridizing fragments given in the centre were calculated from a ³²P-end-labelled *Hind*III digest of λ DNA (lane 1). **b**, DNA isolated from the grandmother (lane 1), mother (lane 2) and two DMD sons (lanes 3, 4) was cleaved with *Bst*XI and the DNA hybridized with pERT87-8 as described in Fig. 1 legend. DNA isolated from an unrelated female heterozygous for the *Bst*XI RFLP is included in lane 5. The darkened symbols above the lanes indicate the DMD trait.

given in Fig. 2 legend). After identifying the pERT87-8 RFLPs, two additional RFLPs were found to be recognized by pERT87-1 (a 1.4-kb *Kpn*I/*Sal*I fragment 16 kb away from pERT87-8; Fig. 1a). Allele frequencies of these RFLP alleles have been determined and mendelian inheritance established. On *Xmn*I digestion, pERT87-1 detects 8.7- and 7.5-kb fragments in heterozygous females (allele frequencies 0.72 and 0.28, respectively). With *Bst*NI digestion, pERT87-1 detects fragments of 3.1 and 2.45 kb/0.65-kb fragments with an invariant 0.7-kb fragment in heterozygous families (allele frequencies 0.52 and 0.48, respectively). One or other of the pERT87-1 RFLP alleles was found to be useful in females from whom no useful information could be obtained using the pERT87-8 *Bst*XI or *Taq*I RFLPs. If there is no linkage disequilibrium of these four RFLPs at the pERT87 region, the expected combined heterozygosity is 0.90. The observed heterozygosity in women studied with all four RFLPs is slightly greater than 0.6. The pERT87-8 region has been mapped⁹ distal to the (X; autosome) translocation breaks within Xp21 in DMD females^{2,3} and should complement the existing proximal probe 754⁵ for use in carrier detection and prenatal diagnosis of DMD. The ability of pERT87-8 and pERT87-1 to distinguish tightly linked RFLP alleles in women at risk for DMD and detection of presumed deletions at the locus make these the most informative probes to date in following the DMD X chromosome.

The deletions detected by pERT87 subclones in the five unrelated DMD males provide a unique tool for defining the extent of the DMD locus. At present, all five individuals lack a minimum of 38 kb of genomic sequences. Expansion of the cloned region by chromosomal 'walking' on total genomic or sorted human X-chromosome bacteriophage libraries²⁰ should elucidate the extent of these deletions. Newly acquired unique-sequence DNA segments can be used to screen for additional deletions, structural rearrangements or new RFLPs. Determination of the common region deleted or rearranged in DMD males should define the exact location of the DNA sequences involved in DMD. Analysis of the cloned DNA from this region should lead to an understanding of the molecular mechanisms that give rise to the phenotype of DMD.

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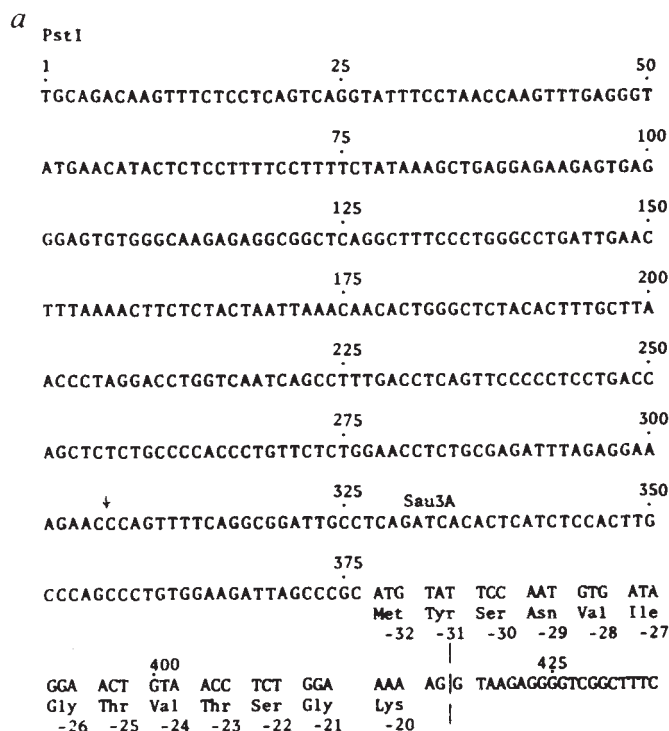
Relationship between an enhancer element in the human antithrombin III gene and an immunoglobulin light-chain gene enhancer

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Enhancers, cis-acting transcriptional control elements have been described in both viral and cellular genes¹. They influence transcription in a quantitative fashion, act over relatively large distances (several kilobases, kb) and behave independently of their position and orientation. Enhancers have been described in immunoglobulin, chymotrypsin and insulin genes²⁻⁹. They bear little homology with each other except for an 8-base pair (bp) 'consensus' core element, GTGG^{AAA}TTT (refs 10, 11), but even this element is sometimes non-homologous⁹. I have searched for such elements in the human antithrombin III (AT-III) gene. AT-III is an important coagulation protein which inactivates thrombin¹². It is produced by the liver and, to a lesser extent, by the kidney¹³. Here, I report that the 5' flanking region of the AT-III gene encodes a segment homologous with the enhancer containing the joining-constant_κ (J_{κ} - C_{κ}) intron of immunoglobulin κ -chain genes. This extensive homology suggests the existence of regulatory factors that recognize common DNA sequences in lymphoid tissues and in those which express AT-III.

Two regions in the 5'-flanking region of the AT-III gene demonstrate sequence homology with sites in the J_{κ} - C_{κ} introns of murine or human immunoglobulin C_{κ} genes (Fig. 1). Region 1 corresponds to nucleotides 252-264 of the AT-III gene¹⁴ and to nucleotides 3,904-3,916 of the J_{κ} - C_{κ} intron of human and murine C genes^{15,16}. Ten of 13 residues (77%) were homologous in the AT-III and human J_{κ} - C_{κ} sequences and 9 of 13 residues (69%) were homologous in the AT-III and murine J_{κ} - C_{κ} sequence. A second, more extensive, region of homology (17 of 22 bases, 77%) was observed between nucleotides 291 and 312



b



Fig. 1 Homologies between the human AT-III 5'-flanking region and human and mouse J_{κ} - C_{κ} large introns. **a**, DNA sequence of the immediate 5' end of the AT-III gene. The sequence shown here is a portion of a 1.2-kb PstI genomic subclone (pAT1.2) containing 304 bp upstream flanking region, 72 bp 5'-untranslated region and codons for the first 13 amino acids of the signal peptide¹⁴. The rest of the subclone contains ~800 bp of intervening sequence. The arrow at position 304 denotes the transcriptional start site. **b**, Sequence homologies between AT-III and J_{κ} - C_{κ} introns. In region 1, the AT-III sequence shown is that between nucleotides 252 and 264. The J_{κ} - C_{κ} sequence is that between nucleotides 3,904 and 3,916, according to the numbering system of Max *et al.*¹⁶. In region 2, the AT-III sequence is that between nucleotides 291 and 312 and the J_{κ} - C_{κ} sequence is that between nucleotides 3,868 and 3,892 (refs 15, 16). Underlined regions correspond to polyoma virus or core enhancer sequence homologies¹⁷⁻¹⁹.

of AT-III and nucleotides 3,869-3,892 of the murine J_{κ} - C_{κ} region, although homology with the human J_{κ} - C_{κ} region was not observed. Although regions 1 and 2 are short and homology could conceivably be fortuitous, it was striking that both regions bear similarities to known viral enhancer elements. For example, in region 1, the underlined area (Fig. 1b) is homologous with a portion of the polyoma virus enhancer TCTCCACCA¹⁷⁻¹⁹. Similarly, the underlined area in region 2 is homologous with the 'core' consensus sequence GTGG^{AAA}TTT, thought to be^{10,11} common to some eukaryotic viral enhancer elements. Thus, the 5'-flanking region of the human AT-III gene might contain functional enhancer activity; I tested this suggestion in an *in vivo* transfection assay using the chloramphenicol

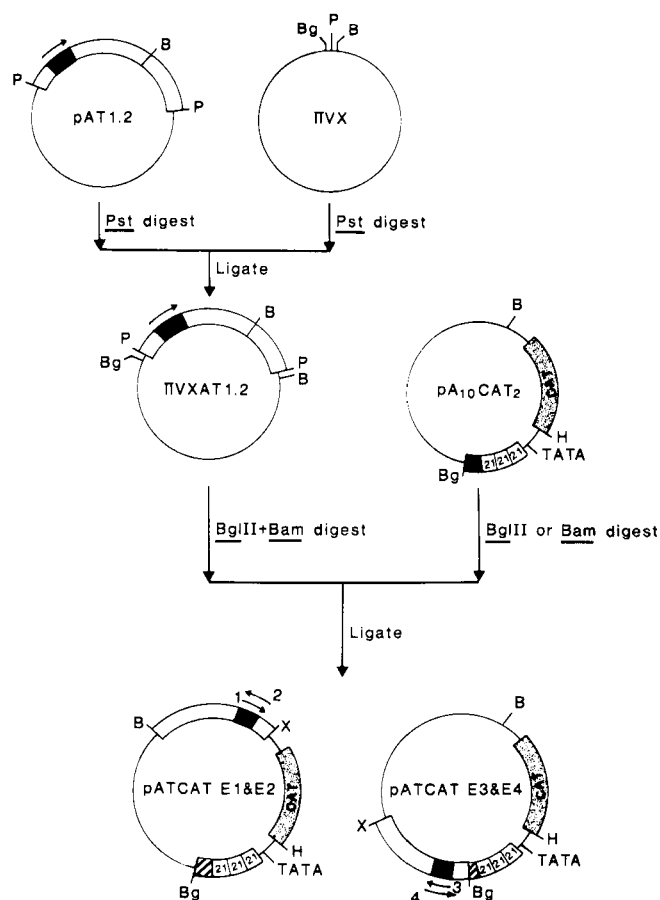


Fig. 2 Construction of AT-III CAT vectors. pAT1.2 is the 1.2-kb AT-III genomic subclone described in Fig. 1 legend. The insert was excised with *Pst*I and ligated into the miniplasmid π VX³⁷ in such an orientation that the region containing the enhancer element was bounded by a 5' *Bgl*II site in the π VX polylinker and the 3' *Bam*HI site in IVS-1 of the AT-III gene. Following digestion of π VXAT1.2 with *Bgl*II and *Bam*HI, the 800-bp-long enhancer-containing fragment was ligated in both possible orientations into the *Bgl*II (upstream) or *Bam*HI (downstream) sites of the enhancerless CAT expression vector pA₁₀CAT₂. The resulting four constructs were designated pATCAT-E1-E4, with only orientations 1 and 3 shown here for simplicity. The arrows indicate the 5' → 3' direction of the enhancer element in each of the constructs. Note that the relative proximities of the enhancer element to the CAT promoter are therefore E3 > E4 > E2 > E1. P, *Pst*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; X, *Bam*HI or *Bgl*II sites destroyed by ligation of nonregenerative GATC sticky ends. Before transfection studies, all plasmids were isolated by the alkaline lysis method³⁸ and doubly banded in CsCl to ensure complete removal of contaminating nucleic acids. Open box, AT-III-derived sequences; solid box, *J*_κ-*C*_κ enhancer-like region; solid lines, plasmid-derived sequences; stippled box, CAT gene; cross-hatched box; truncated SV40 enhancer region; 21, SV40 21-bp repeats.

acetyltransferase (CAT) assay²⁰. Restriction fragments containing the putative enhancer element from the AT-III gene were cloned into the enhancerless vector pA₁₀CAT₂ (Fig. 2) and their effect on transcription quantitated by examining cell extracts for the presence of CAT following transfection. A positive control plasmid, pSV₂CAT, contains a simian virus 40 (SV40) enhancer and directs the synthesis of large amounts of CAT. Each plasmid was transfected into four different eukaryotic cell lines, two of which, the COS-1 African green monkey kidney line²¹ and the Alexander human hepatoma line²² are derived from tissues that normally express AT-III (ref. 13). Cell extracts were prepared and assayed for CAT activity 48 h later²⁰. CAT activities were normalized to the results of Hirt extracts²³ to allow for variations in the transfected DNA uptake and replication. The results (Fig. 3) can be summarized as follows: (1) CAT activity was significantly enhanced above background by the

presence of 800 bp of AT-III gene sequence containing the *J*_κ-*C*_κ homologies; (2) the level of enhancement was generally in inverse proportion to the distance of the putative enhancer element from the SV40 early promoter, although some variability was seen between experiments; (3) enhancer activity was independent of the orientation of the AT-III sequences; (4) enhancer activity was seen only in those cells derived from tissues that naturally produce AT-III, that is, COS-1 (kidney) and Alexander hepatoma (liver). Examination of Hirt extracts of transfected cells showed only small differences in plasmid copy number within any single experiment, indicating that the increased CAT activity was not the result of preferential replication of enhancer-containing constructs in COS-1 cells or of variability in DNA uptake (my unpublished data). Therefore, I conclude that the 5' end of the human AT-III gene contains sequences that potentiate heterologous gene transcription in a tissue-specific manner when assayed by transient expression.

The extensive sequence homology observed between AT-III and immunoglobulin *J*_κ-*C*_κ enhancers suggested that there is also functional homology. This possibility was tested by introducing a murine immunoglobulin *J*_κ-*C*_κ enhancer into Alexander and COS-1 cells and the AT-III enhancer into a murine myeloma line and measuring CAT activity 48 h later. In the former case, the immunoglobulin *J*_κ-*C*_κ enhancer element was cloned into the *Bgl*II site of pA₁₀CAT₂. The resultant plasmid, designated pA₁₀CAT₂-κ, was then introduced into Alexander or COS-1 cells by calcium phosphate precipitation. The plasmids pA₁₀CAT₂-κ and pATCAT-E3 were transfected into 10⁷ M12 murine myeloma cells using the electroporation technique²⁴ and cell extracts were assayed for CAT after 48 h (Table 1). As observed previously, the AT-III enhancer functioned in both Alexander and COS-1 cells. In a similar fashion, pA₁₀CAT₂-κ also functioned above background when introduced into M12 cells. More significant, however, was the observation that the AT-III enhancer functioned in M12 cells and that the immunoglobulin *J*_κ-*C*_κ enhancer functioned in at least one of the cell lines (COS-1) derived from AT-III-producing tissues. CAT activity was generally less in the heterologous cells: the AT-III enhancer functioned better in COS-1 and Alexander cells whereas the *J*_κ-*C*_κ enhancer functioned better in M12 cells. pA₁₀CAT₂-κ functioned at only

Table 1 Relative activity of AT-III and *J*_κ-*C*_κ enhancers in myeloma cells, hepatocytes and COS-1 cells

Transfected plasmid	M12 myeloma	CAT activity in Alexander hepatoma	COS-1
pATCAT-E3	4.0	4.3	12.8
pA ₁₀ CAT ₂	7.2	1.5	4.4
pA ₁₀ CAT ₂ -κ	1.0	1.0	1.0

Plasmid pATCAT-E3 is described in Fig. 2 legend. pA₁₀CAT₂-κ was constructed by removal of the 1.25-kb *Hind*III/*Hpa*I fragment from the rearranged murine gene MOPC-41κ¹⁶. The fragment was repaired with DNA polymerase (Klenow fragment) and deoxynucleoside triphosphates. *Bgl*II linkers were added, followed by ligation into the *Bgl*II site of pA₁₀CAT₂. The orientation was such that the former *Hind*III site was brought closer to the SV40 early promoter. 20 μg of each plasmid was introduced into either Alexander or COS-1 cells by standard calcium phosphate DNA co-precipitation onto 5 × 10⁵ cells plates the day before. For M12 cells, transfection was accomplished by electroporation of 10⁷ myeloma cells in 0.5 ml phosphate-buffered saline (PBS). The procedure was essentially as described previously²⁴ using an Isco Model 494 electrophoresis power supply. Voltage was adjusted to 2,000 V, current to 0.9 mA and the adjustable current and wattage set at a minimum. Cells were kept at 0 °C in PBS during electroporation and for 10 min afterwards before being replated in modified Eagle's medium (NEM)/10% fetal calf serum (FCS). Following transfection, each cell line was grown for 48 h in MEM/FCS, at which time CAT activity was measured in extracts. The values shown represent the increase over the amount of CAT conversion seen after transfection with pA₁₀CAT₂ alone (value arbitrarily set at 1).

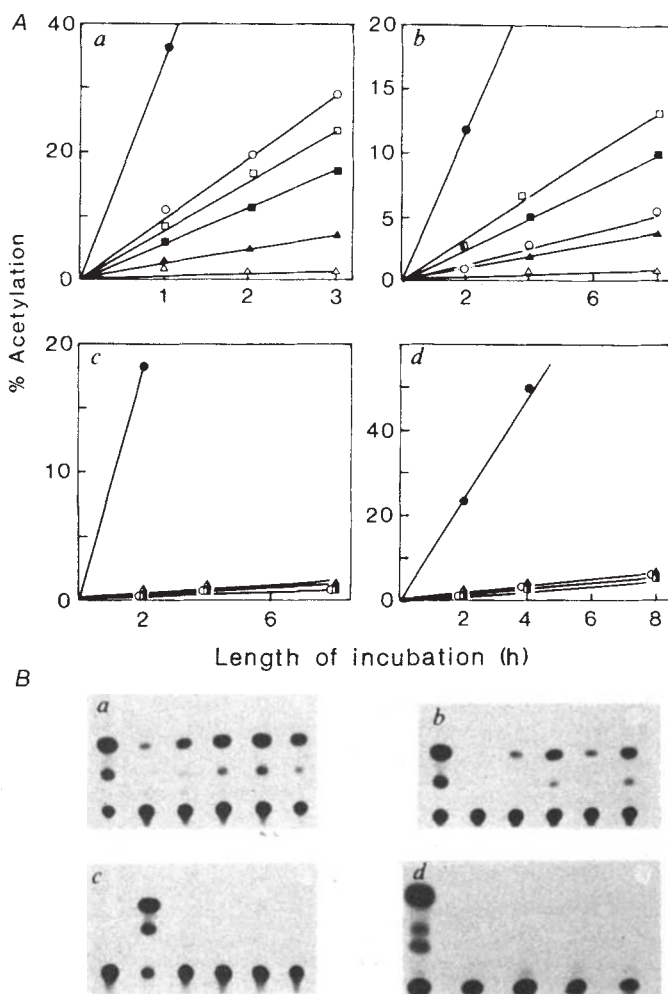


Fig. 3 Tissue-specific CAT expression. **A**, Kinetics of CAT activity in four different cell lines: *a*, COS-1; *b*, Alexander human hepatoma; *c*, HeLa; *d*, mouse thymidine kinase-negative L cells. Transfections were with pSV2CAT (●); pA₁₀CAT₂ (Δ); pATCAT-E1 (▲); pATCAT-E2 (■); pATCAT-E3 (○); pATCAT-E4 (□). All points in this and subsequent experiments are normalized for differences in plasmid copy number as determined by DNA dot-blots of Hirt extracts²³. **B**, Thin-layer chromatograms of CAT conversion products. Spots nearest the bottom represent unacetylated ¹⁴C-chloramphenicol. *a*, COS-1; *b*, Alexander; *c*, HeLa; *d*, L cells. From left to right in each lane, the products from transfections with pSV2CAT, pA₁₀CAT₂, pATCAT-E1, pATCAT-E2, pATCAT-E3 and pATCAT-E4, respectively, are spotted. In *c* the pA₁₀CAT₂ and pSV2CAT lanes have been reversed. Each set of experiments was performed at least twice with three different plasmid preparations.

Methods. All cells were grown in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg ml⁻¹ streptomycin and 100 U ml⁻¹ penicillin G. Cells were split into 100-mm tissue culture plates at 5 × 10⁵ cells per plate and 24–48 h later, 20 μg of each of the four AT-III CAT constructs shown in Fig. 2 were introduced into the cells by calcium phosphate precipitation³⁹. As negative and positive controls, pA₁₀CAT₂ or the SV40 enhancer-containing plasmid pSV2CAT were also used²⁰. Precipitates were kept on the cells for 5 h, after which the cells were washed with MEM and subjected to a 25% glycerol/MEM shock for 1 min; 48 h later, cells were washed in Tris-buffered saline, and scraped from each plate. An aliquot was used for Hirt extracts²³. Hirt supernatants were dot-blotted onto nitrocellulose⁴⁰ and hybridized with a nick-translated pAT1.2 insert (specific activity 3 × 10⁸ d.p.m. μg⁻¹). Hybridizations were carried out overnight at 68 °C in a solution consisting of 6 × SSC, 5 × Denhardt's solution, 1% SDS, 10 mM phosphate buffer pH 7.0, 1 mM EDTA, 100 μg ml⁻¹ sonicated salmon sperm DNA, 10% dextran sulphate (Pharmacia) and 10⁶ d.p.m. ml⁻¹ of nick-translated probe. The remaining cells were subjected to one cycle of freeze-thawing and then sonicated in 100 μl 250 mM Tris-HCl, pH 7.8. In the case of COS-1 cells, 20 μl aliquots of clarified extract were assayed for CAT activity, whereas in all other cases 50 μl was used. The assay system was that described by Gorman *et al.*²⁰, which relies on the conversion of ¹⁴C-chloramphenicol to its acetylated derivatives and their resolution by silica gel TLC.

modest levels above background when introduced into Alexander cells. These experiments suggested that two unrelated genes can be expressed in the same tissues if they share homology in enhancer regions.

It was possible that the effects seen with the AT-III or immunoglobulin gene fragments were the result of the introduction of cryptic promoter elements into the pA₁₀CAT₂ vector. To address this issue, I performed a series of primer extension experiments. An end-labelled CAT-specific *Eco*RI/*Pvu*II restriction fragment was strand-separated and hybridized with total RNA from COS-I cells that had been transfected with CAT plasmids 48 h previously. The DNA/RNA hybrid was then extended with reverse transcriptase and the size of the product analysed by polyacrylamide gel electrophoresis (Fig. 4). Transcripts initiating at the SV40 early promoter should have extended products 307 bp in length, whereas those originating from upstream sequences should be longer. Transcripts initiated correctly in each of the plasmids tested regardless of whether they contained SV40, AT-III or immunoglobulin upstream sequences.

An understanding of the developmental and/or tissue-specific control of eukaryotic gene expression will ultimately require an appreciation of the various factors whose interactions influence transcription. Specific DNA sequences are known to affect gene transcription, presumably by serving in a manner analogous to classical prokaryotic promoters in allowing for binding of RNA polymerases^{25–29}. The more recent descriptions of enhancers have defined a new class of transcriptional control signals, often with tissue-specific regulatory capacity. That enhancers function irrespective of position and orientation^{1,27–29} has given rise to the notion that, by disrupting chromatin structure, they serve as preferred entry points for RNA polymerases which then scan adjacent DNA sequences until encountering a promoter ele-

ment^{30–32}. Here, I provide evidence for an enhancer element at the 5' end of the human AT-III gene. Its presence was initially inferred on the basis of homologies between the AT-III gene and the *J_κ-C_κ* introns of murine and human immunoglobulin genes which contain tissue-specific enhancers. In addition to their homologies to viral sequences, however, the AT-III and immunoglobulin *J_κ-C_κ* genes share more extensive homologies with one another. That these homologies occur in close physical association with the polyoma virus-like and viral core sequences suggests that they are involved in the tissue-specific regulation of AT-III expression.

Immunoglobulin genes function poorly, if at all, in nonlymphoid cells such as fibroblasts^{3,4}. However, because of the close structural homology between AT-III and *J_κ-C_κ* enhancers, I tested for functional homology by introducing a murine *J_κ-C_κ* enhancer into Alexander hepatoma cells and the AT-III enhancer into myeloma cells. In both cases increased CAT activity was seen although the magnitude of the enhancement was low. This may reflect a suboptimal interaction between the enhancers and tissue-specific factors³³ or the need for additional factors that are absent from the heterologous cells. The *J_κ-C_κ* enhancer showed low activity even in myeloma cells and is consistent with the findings of Picard and Schaffner⁵ who demonstrated that the murine *κ*-chain gene enhancer is only 5% as effective as heavy-chain gene enhancers in promoting heterologous gene transcription.

Extensive homologies among enhancers have not previously been reported. Laimins *et al.*¹⁰ and Weiher *et al.*¹¹ have noted a consensus core sequence (GTGG^{AAA}_{TTT}G) in several viral enhancer elements but additional sequence homology was not observed. Walker *et al.*⁹ reported that the sequence GTGGAAA was found in fragments with enhancer activity from the 5'-flanking region of human and rat insulin genes although the

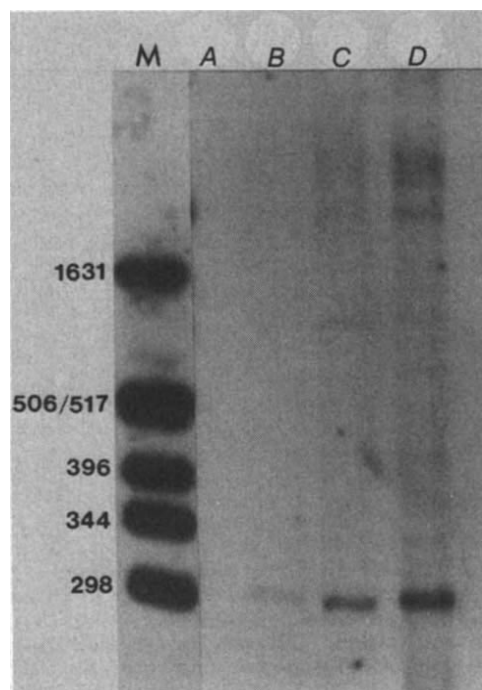


Fig. 4 A primer extension analysis of CAT transcripts in COS-1 cells. M, Marker. Primer extended products from cells transfected with: A, pA₁₀CAT₂; B, pA₁₀CAT₂-K; C, pATCAT-E3; D, pSV₂CAT. **Methods.** Transfections were performed with 20 µg per plate of each plasmid tested (pA₁₀CAT₂, pSV₂CAT, pATCAT-E3 and pA₁₀CAT₂-K). Cells were collected after 48 h and total RNA was extracted by the guanidine hydrochloride method³⁹. 25 µg RNA was hybridized with 80,000 d.p.m. of a 104-nucleotide-long EcoRI/PvuII strand-separated restriction fragment from the CAT gene which had been labelled at the EcoRI site to specific activity of 2 × 10⁷ d.p.m. µg⁻¹ using polynucleotide kinase and [³²P]ATP (specific activity 7,500 Ci mmol⁻¹; Amersham). Hybridization conditions were: 80% formamide, 280 mM NaCl, 60 mM PIPES, pH 6.8 in 10-µl reactions at 51 °C for 16 h. Hybrids were then phenol-extracted, precipitated and redissolved in 25 µl 70 mM KCl, 10 mM Tris, pH 8.2, 8 mM MgCl₂, 10 mM dithiothreitol, 0.25 mM of each deoxynucleoside triphosphate and 50 U avian myeloblastosis virus reverse transcriptase. After 60 min at 42 °C, the reactions were phenol-extracted, precipitated, redissolved in formamide dye buffer and electrophoresed through an 8% polyacrylamide/8 M urea sequencing gel.

sequence was not required for optimal activity. Therefore, the homology between AT-III and J_K-C_K enhancers is striking in view of the minimal relatedness of other enhancers.

In an effort to correlate more precisely the J_K-C_K homology with enhancer activity, I have tested a series of deletion plasmids, one of which contains only 177 bp of AT-III sequence, including the immunoglobulin enhancer homology (nucleotide 153-330; Fig. 1). When tested in COS-1 cells, this construct is as active as any of the constructs shown in Fig. 2 (unpublished). Current experiments are focused on the construction of additional deletions to determine whether the same or different sequences are required for full expression in COS and Alexander cells.

The significance of the homologies reported here is not yet clear. It may be that cellular genes contain only a limited number of enhancer elements many of which have fairly extensive homology with each other despite widely different functions of the genes they control. AT-III and light-chain genes might fortuitously represent members of one such subset of enhancers. Alternatively, as B cells are thought to originate in the liver during early embryogenesis^{34,35}, these enhancers might represent common tissue- or stage-specific targets for cellular transcription factors. A distinction between these and other alternatives should be possible when more cellular enhancers have been characterized. In either case, it seems unlikely that the

homologies noted here represent the entire region responsible for tissue specificity. Despite the fact that the AT-III and J_K-C_K enhancer elements can function in heterologous cells, it is clear at least for immunoglobulin genes that this is not normally the case³⁶. Other unique cellular factors and/or DNA sequences are likely to be responsible for conferring additional tissue specificity on gene expression.

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Local cytoplasmic calcium gradients in living mitotic cells

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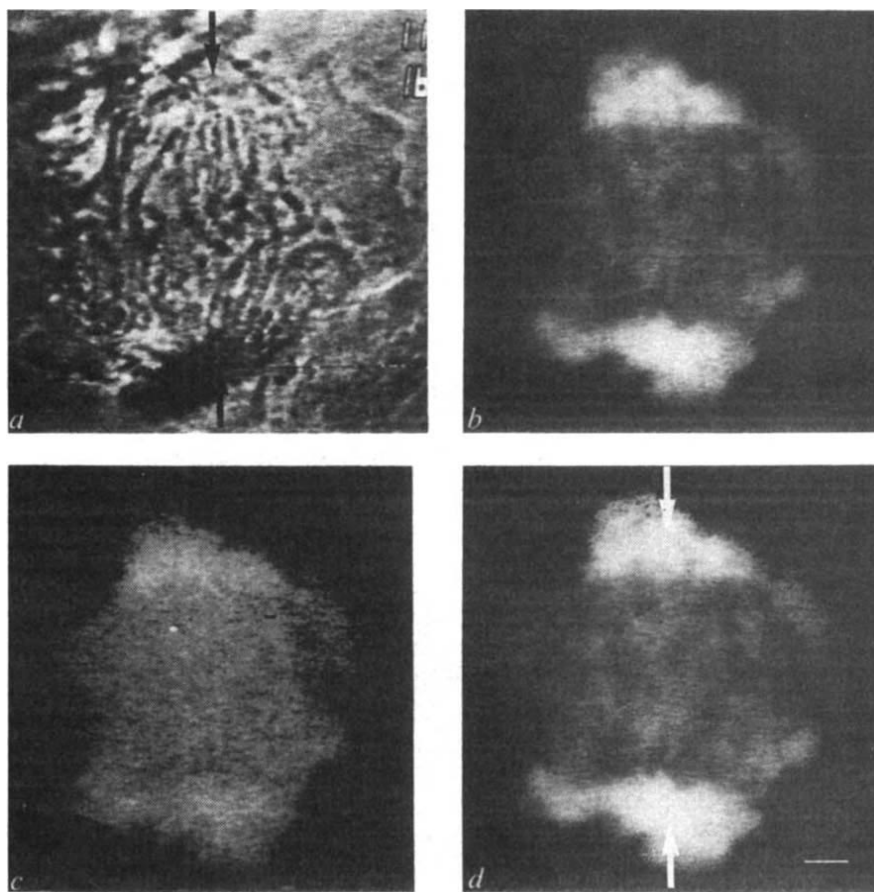
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Cytoplasmic free calcium has been proposed as a regulator of many microtubule-mediated processes, including mitosis. It has been difficult to test this hypothesis because methods for local measurement of free Ca²⁺ in the living cell have not been available. We have used the fluorescent calcium indicator dye Quin-2 (methoxyquinoline-1bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), which allows such observations to be made by digital processing of fluorescent images from the light microscope. Here

Fig. 1 Localization of calcium at the mitotic spindle poles of endosperm cells during mitosis. *a*, Bright-field image of a cell in anaphase; *b*, *c*, the same cell observed with 340 and 360 nm excitation after loading with Quin-2; *d*, the I_{340}/I_{360} ratio calculated at each point and redisplayed as an image of the ratio values. Bright areas in *d* correspond to areas of high $[Ca^{2+}]$. Cell wall-free endosperm cells of *Haemanthus Katherinae Baker* were expressed from young ovules onto glass coverslips coated with a thin film of Phytoagar (Gibco), overlain with a thin film of Gelrite (Kelco)²⁷ and perfused with 10 mM citrate buffer, pH 5.2, containing 1 mM $MgCl_2$, 3% glucose and 30 μM Quin-2/AM, for 20 min. Parallel experiments were carried out in medium supplemented with 0.1 mM $CaCl_2$. After loading, the cells were rinsed and incubated in perfusion buffer without the dye for 15 min and cells observed on a Diavert microscope (Leitz) modified for near-ultraviolet excitation¹⁴. Fluorescence-excitation light was passed through narrow band-pass filters centred at 340 and 360 nm, and emitted light was passed through a 500-nm broad bandpass filter (Oriel). (The image-analysis system has been described previously²⁸.) Fluorescent images were obtained with a Dage-MTI model 65 SIT image-intensifier video camera and were recorded on a Panasonic NV8030 videotape recorder with the automatic gain control suppressed. The camera was modified to linearize the intensity response. Images were digitized using a CAT-800 image digitizer (Digital Graphics) housed in a Horizon microcomputer (North Star Computers). The digitizer divides the image into 484×512 picture elements (pixels) and assigns intensity levels from 0 to 255 using a high speed A to D converter. Six video frames were averaged for each image to reduce noise, and the averaged images were transferred to an LSI 11/23 microcomputer (Digital) with a Gould IP8400 image analyser for further processing. Background values were determined by digitizing images of regions which contained no cells and subtracting these images pointwise from the images of cells. Cell-associated autofluorescence was $<10\%$ of the Quin-2 fluorescence with 340 nm excitation. The background-corrected images were then divided pointwise to provide an I_{340}/I_{360} image. The regions corresponding to the cell were identified using a threshold intensity in the 360 nm image, and the I_{340}/I_{360} image of the cell is shown in *d*. The areas of high cytoplasmic Ca^{2+} (high values of I_{340}/I_{360}) correspond to the mitotic spindle poles (arrows). The average cytoplasmic $[Ca^{2+}]$ is ~ 2 – 3 -fold higher at the spindle poles than in the interchromosomal region. Similar values were observed in 19 other anaphase cells. Scale bar, 100 μm .



we report the application of this technique to the study of local Ca^{2+} concentrations in mitotic endosperm cells of *Haemanthus* sp., and show that there is transient increase in free Ca^{2+} at the mitotic spindle poles during anaphase. This locally high Ca^{2+} may provide a mechanism for the regional control of microtubules and other cytoskeletal elements during anaphase.

Despite intensive study, the mechanisms of cell division remain poorly understood¹⁻³. Actin filaments⁴ or microtubules⁵ have been suggested as mediators of chromosome movement during mitosis, but the precise role of these cytoskeletal elements is unclear. Several lines of evidence suggest that cytoplasmic Ca^{2+} is important in the regulation of mitosis. Conditions that lower cytoplasmic Ca^{2+} delay the onset of anaphase⁶, and microinjection of Ca^{2+} during metaphase stimulates the transition to anaphase⁷. During anaphase, the spindle pole-to-chromosome microtubules become shorter; in echinoderms these spindle microtubules are very sensitive to Ca^{2+} during anaphase⁸. Calmodulin increases the sensitivity of microtubules to Ca^{2+} both *in vitro*⁹ and *in vivo*¹⁰, and high concentrations of calmodulin have been observed in the mitotic spindle of animal^{11,12} and plant¹³ cells during anaphase. These data suggest a regulatory role for Ca^{2+} in the shortening of the half-spindle, possibly acting through its effect on microtubules.

To determine whether cytoplasmic Ca^{2+} acts as a regulator of mitosis, accurate measurements of changes in Ca^{2+} must be made at each stage of mitosis. Because of the difficulty in obtaining tightly synchronized populations of dividing cells, such measurements must be made on individual cells assigned to a stage of mitosis by direct observation at the time of measure-

ment. Recently, a method has been developed for the measurement of cytoplasmic Ca^{2+} in individual cells using the fluorescent dye Quin-2 (ref. 14). The method is based on the observation that Quin-2 fluorescence excited at 340 nm is highly Ca^{2+} dependent, whereas the Quin-2 fluorescence excited at 360 nm is independent of $[Ca^{2+}]$ (ref. 15). Thus, the ratio of the two intensities (I_{340}/I_{360}) is dependent on Ca^{2+} but independent of changes in dye distribution resulting from changes in cell shape and thickness during mitosis. We have used this method to measure the average cytoplasmic $[Ca^{2+}]$ in rat kangaroo epithelial cells. During mitosis, the average cytoplasmic $[Ca^{2+}]$ was half the interphase value (28 against 53 nM), with no statistically significant variations observed among the mitotic stages¹⁶. These measurements of average $[Ca^{2+}]$ indicated that cytoplasmic Ca^{2+} does change during mitosis, but no evidence was obtained concerning local changes in $[Ca^{2+}]$ which might be required for directed motion. Here we report measurements of local $[Ca^{2+}]$ in mitotic cells.

The intensity ratio method is well suited for measuring local $[Ca^{2+}]$ by image digitization and analysis (see Fig. 1 legend). The intensity values provide a two-dimensional representation of cytoplasmic $[Ca^{2+}]$ within the cell. The horizontal resolution of the method is limited by the thickness of the cell and the optical properties of the microscope. Although the method has been developed using Quin-2, it is suitable for use with dyes of higher quantum efficiency and more favourable spectroscopic qualities, such as Fura-2 (ref. 17).

We used endosperm cells of the African blood lily, *Haemanthus Katherinae Baker*. The cells are extremely large, facilitating

observation of gradients, and their mitosis has been studied extensively¹⁸. The acetoxymethylester of Quin 2 (Quin-2/AM) was added to the cells (Fig. 1), which trapped Quin-2 by conversion of the ester to the tetracarboxylic acid. Cell-associated fluorescence increased ~10-fold with 340 nm excitation and 492 nm emission. During loading, the chromosome movement seems to stop. However, this inhibition is found to persist only for 5 min after removal of Quin-2/AM, after which anaphase progresses normally. For cytoplasmic Ca^{2+} measurements, the cells were incubated in buffer lacking Quin-2/AM for at least 15 min before the observation. Based on observation of several hundred cells, the loading procedure does not block the progression of cells from metaphase to telophase.

Criteria for selection of cells for measurements included: (1) flatness; (2) low refractive index; (3) no starch grains; (4) no debris or other cells covering the cell of interest; (5) observable chromosome movement in anaphase cells. Cells were observed, and I_{340}/I_{360} ratio images obtained, at all stages of mitosis. In interphase cells, the cytoplasmic $[\text{Ca}^{2+}]$ is fairly uniform. However, the cytoplasm of the ratio image is 1.6 times brighter than the nucleus. It is not clear that this can be attributed to a lower $[\text{Ca}^{2+}]$ in the nucleus as the behaviour of Quin-2 in the nuclear environment cannot be readily predicted. It is unlikely that the lower value results from exclusion of dye from the nucleus; when cells are labelled in prometaphase and allowed to proceed through telophase, the newly formed daughter nuclei also have a lower I_{340}/I_{360} fluorescence ratio. The prometaphase cell also exhibits a low ratio in the area of the nucleus despite the breakdown of the nuclear membrane. Cytoplasmic free Ca^{2+} is still evenly distributed. The metaphase cell does not show any readily observed inhomogeneity of $[\text{Ca}^{2+}]$ either along the spindle itself or outside the spindle area.

In anaphase, there is a marked departure from the homogeneous distribution of free Ca^{2+} seen in the previous stages. In 24 out of 28 anaphase cells examined, we observed regions of higher free Ca^{2+} near the mitotic spindle poles (Fig. 1). This distribution is observed in cells in early, mid and late anaphase, indicating that the gradient lasts from 30 to 45 min. Quantitative analysis in 20 of these cells indicated that the I_{340}/I_{360} ratio at the polar region was 1.67 ± 0.08 (s.e.m.) $\times$ higher than the ratio in the region between the chromosomes. This contrasts sharply with metaphase where the I_{340}/I_{360} ratio at the poles was only 1.1 ± 0.04 higher than at the metaphase plate. Eight anaphase cells were excluded from quantitative analysis for the following reasons: (1) Four cells, which did not demonstrate localization at the poles, were not flat. As the thickness of the cell becomes greater, contributions from out-of-focus parts of the cell may interfere with local measurements of intracellular Ca^{2+} ; (2) Four other cells which demonstrated localization of Ca^{2+} at the spindle poles were excluded because the Quin-2 fluorescence was either too high or too low for accurate measurements. In telophase, the cytoplasmic free Ca^{2+} distribution is once again more homogeneous, with lower I_{340}/I_{360} in the daughter nuclei.

To investigate the possibility that in cells loaded with Quin-2, citrate, a Ca^{2+} chelator, caused changes in cytoplasmic distribution, we compared cells incubated in low Ca^{2+} and Ca^{2+} supplemented medium (0.1 mM). In all stages of mitosis the pattern for that stage when incubated in high Ca^{2+} was not significantly different from that of the cells incubated in low Ca^{2+} medium. Sixteen anaphase cells were observed in low Ca^{2+} medium and the intensity ratio at the poles to that of the mid-zone was 1.66 ± 0.07 . Four additional anaphase cells were observed in high Ca^{2+} , and the ratio of Quin-2 fluorescence at the poles to that of the mid-zone (1.68 ± 0.27) was not significantly different from that of the low Ca^{2+} medium. In parallel experiments, whole-cell values of cytosolic Ca^{2+} in cells incubated in high Ca^{2+} medium (52 ± 7 nM, $n=4$) were similar to cells incubated in low Ca^{2+} medium (47 ± 4 nM, $n=11$). These observations

support the existence of large stores of intracellular Ca^{2+} and the existence of efficient Ca^{2+} homeostatic mechanisms in *Haemanthus endosperm*.

Our data indicate that cytoplasmic Ca^{2+} is elevated in the cytoplasm near the spindle poles during anaphase. Membrane-limited organelles have been observed along the mitotic spindle^{19,20} which may sequester Ca^{2+} , presumably to release it during anaphase^{21,22}. Our data show that the rate of release into the cytoplasm (and removal elsewhere) is sufficient to maintain a twofold gradient of $[\text{Ca}^{2+}]$ across the cell. Higher concentrations are probably attained near the sites of release, but our measuring procedure averages I_{340}/I_{360} throughout the thickness of the cell. The effect of Quin-2 itself on the gradient is not known, but Quin-2 probably reduces the magnitude of the $[\text{Ca}^{2+}]$ gradient by acting as a Ca^{2+} chelator.

Although the effect of local Ca^{2+} gradients during anaphase is not yet known, certain hypotheses can be suggested. Binding of Ca^{2+} reduces the mobility of calmodulin in the cell¹⁰, probably by causing binding of Ca^{2+} -calmodulin to relatively immobile proteins. Thus, locally high Ca^{2+} may trap calmodulin in the mitotic spindle, leading to the observed inhomogeneous distribution of calmodulin during anaphase¹¹⁻¹³. Ca^{2+} -calmodulin causes disassembly of microtubules^{9,10}, and the high concentrations of Ca^{2+} and calmodulin would be expected to cause the shortening of the kinetochore fibres as the chromosomes approach the poles. Of course, Ca^{2+} is also a regulator of actin-based force-generating systems²³, and locally high Ca^{2+} may activate such a system to provide a motile force.

Transient intracellular Ca^{2+} gradients have been reported for several phenomena, ranging from nerve-cell depolarization²⁴ to sea urchin egg activation²⁵. We have shown that long-lasting Ca^{2+} gradients exist in dividing cells during anaphase which may provide a mechanism for local control of motility within the cells. For example, microtubules could be disassembled by Ca^{2+} calmodulin in one region (for example, the mitotic spindle) while microtubule assembly proceeds elsewhere (for example, the interpolar microtubules)²⁶. These studies are now being extended to other cell types, particularly mammalian cells.

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